CHARACTERIZATION OF MYCOBACTERIA SPP. AND ANTIMYCOBACTERIAL ACTIVITIES OF PLANT DERIVED COMPOUNDS FROM ANACARDIACEAE FAMILY

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ANACARDIACEAE **DT&H No.:** 4594010

Searsia undulata (Jacq.) T.S.Yi, A.J.Mill. & J.Wen

Locality: 25°44' S, 28°16' E 2528CB

Gauteng, Pretoria, National Botanical Garden

Notes:
Tree. PhD Project: Antimycobacterial activity of leaf extracts from selected plants of Anacardiaceae.

Veg. Type: On ridge behind buildings.

Collector: Kayoka-Kabonga, P.,
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Exact wording of the title of the thesis as appearing on the copies submitted for examination:

CHARACTERIZATION OF MYCOBACTERIA SPP. AND ANTIMYCOBACTERIAL ACTIVITIES OF PLANTS DERIVED COMPOUNDS FROM ANACARDIACEAE FAMILY

I declare that the above thesis is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

Signed 8/02/2017

SIGNATURE DATE

DEDICATION

This research is dedicated to all tuberculosis sufferers. I also dedicate this work to my late father, Raphael Kabongo for teaching me resilience and work ethics, to my mother, Therese Kabongo for teaching me the values that are leading my life and to my son, Axel Kayoka, and daughter, Benissa Kayoka, my best friends; you have been part of my life's goals motivation from the time you were born up to date. May this work inspire you to persevere in reaching your goals and live your dreams to the mutual benefit of communities.

Happy is the man who finds wisdom and the man who gains understanding: Proverbs 3:13.

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ABSTRACT

The treatment of tuberculosis (TB) is currently a challenge due to multi- and extensively drug resistant strains of *Mycobacterium tuberculosis*. *Mycobacterium bovis* and *M. tuberculosis* cause clinically indistinguishable tuberculosis in humans. Both *M. bovis* and *M. tuberculosis* have been isolated from humans and animals. Plant species contain antimicrobial compounds that may lead to new anti-TB drugs. To conduct *in vitro* antimycobacterial assays, it is important to include current clinical isolates as new strains of bacteria might be circulating under the ongoing climate change environment. The overall goal and objectives of this study were to isolate and characterize mycobacteria species from South Africa, to test some selected plant species of the Anacardiaceae family for antimycobacterial activity using some of the newly isolated and reference strains of mycobacteria followed by cytotoxicity evaluation of the most active plant species, and finally the isolation and characterization of at least one compound from the most active and least toxic plant.

This study led to the discovery of a new isolate of *Mycobacterium Avium Complex species* from black wildebeest. Other non-tuberculous mycobacteria and *M. bovis* isolates were identified from other animal species. Five out of 15 plant species screened showed good activity against *Mycobacterium* species. Five antimycobacterial compounds were isolated from *Searsia undulata*, the most active plant species. Two out of the five compounds were identified, and one compound appears to be novel, but both compounds have been isolated for the first time from *Searsia undulata*. An incidental finding was the potential anticancer property of extracts of *Searsia undulata*.

Recommended future activities include isolation and identification of more active compounds from *Searsia undulata* which were visible in bioautography analysis, as well as synergy evaluation of antimycobacterial activities of the different compounds with current anti-tubercular drugs.

Key words: **Characterization, Antimycobacterial, NTM mycobacteria, Black wildebeest, MDR-*M. bovis*, *MDR-M. tuberculosis, Anacardiaceae, Searsia undulata, Betulonic acid.***

The project involved the following steps:

Isolation and characterization of *Mycobacterium* species

A total of 80 samples from 44 animals were processed, and included samples from 12 black wildebeest (*Connochaetus gnou*) (n=26); 10 cattle (*Bos taurus*) (n=31), 1 impala (*Aepyceros melampus*) (n=1); 1 rabbit (*Oryctolagus cuniculus*) (n=2) and 20 warthog (*Phacochoerus africanus*) (n=20). These samples namely lymph nodes, liver, lung and kidney were obtained from slaughtered animals showing lesions suggestive of tuberculosis and positive reactors to tuberculin test (cattle only). The methods used were isolation in liquid medium using the BACTEC^{TM} MGIT^{TM} 960 system, solid media and Löwenstein Jensen slants, with glycerol and pyruvate. The isolates were further identified using the commercial kit GenoType CM/AS reverse line blot assay and DNA strip *Mycobacterium* identification species (Hain Life Science, Gmbh Nehren, Germany). The isolates were further characterized by multiplex PCR, spoligotyping, Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR), gene sequencing, phylogenetic analysis and antimicrobial susceptibility test of cattle isolates (*M. bovis)* against first line TB drugs using a Genotype MTBDRplus kit (Hain Life Science GmbH, Nehren, Germany).

The samples from cattle yielded 15 isolates of *M. bovis*; 8 out of the 15 isolates (53%) were resistant to isoniazid (INH) and rifampin (MDR-*M. bovis*), whereas 7 out of 15 (47%) were sensitive to both drugs. The presence of MDR-*M. bovis* is of concern as *M. bovis* causes tuberculosis in humans which is clinically indistinguishable from TB caused by *M. tuberculosis*. Added to this, there are very few data available in South Africa reporting human tuberculosis caused by *M. bovis*. The isolates from cattle were genotyped and yielded two spoligotypes, namely SB0121 (67%) and SB 1235 (33%). The VNTR was type 1. These isolates were from the same origin and most likely belonged to the Kruger National Park cluster as reported by Hlokwe *et al*. 2014.

The first batch of samples, received in February 2009, from black wildebeest yielded non-tuberculous mycobacteria: a novel *Mycobacterium Avium* Complex species was confirmed by gene sequencing and phylogenetic analysis and the second batch, received in August 2010, yielded *Mycobacterium avium* subspecies *hominissuis (M.*

avium subsp*. hominissuis)*. The sample from a rabbit also yielded *M. avium* subsp*. hominissuis*. These findings were of concern as meat from black wildebeest and rabbit are used for human consumption. Non-tuberculous mycobacterial species are among emerging mycobacteria of increasing interest as their clinical significance has been established in humans but their level of involvement has not yet been described in animals. Additionally, some of the members of MAC have been reported as potentially zoonotic which includes *M. avium* subsp. *hominissuis*. Further investigations are needed to fully establish the pathogenicity of non-tuberculous mycobacteria in animals and thus the depositing of new isolates at the international gene data bank is critical to these endeavours.

Samples from warthogs yielded a mixture of *M. bovis* and non-tuberculous mycobacteria, namely *M. intracellulare* and *M. avium*. The sample from impala yielded *M. bovis.* These findings confirm that mixed infection of tuberculous and non-tuberculous mycobacteria occurs naturally.

Quantitative antimycobacterial activity of selected plants, fractions and isolated compounds

In seeking for alternative drugs for the treatment of tuberculosis, plant species are easily accessible natural resources that may contain antimicrobial compounds that could lead to new anti-TB drugs. Previous preliminary screening of some tree species from the Anacardiaceae family showed that many of these species had good activity against *M. smegmatis*, justifying further investigations on their antimycobacterial activity. Leaves from 15 Anacardiaceae tree species were collected, dried and extracted with acetone as a solvent which extracts plant compounds of varying polarities. The leaf extracts were then screened for antimycobacterial activity using a two-fold serial microdilution assay against the pathogenic multidrug resistant *M. bovis* and *M. tuberculosis* and rapidly growing mycobacteria, namely *M. smegmatis*, *M. fortuitum* and *M. aurum*. The vaccine strain, *M. bovis* BCG and an avirulent strain of *M. tuberculosis* H37Ra were also used. Extraction yield ranged from 0.8% to 18.8%. The highest percentage yield was observed with *Searsia magaliesmontana* (18.8%) followed by *S. undulata* (12.5%) and *Protorhus longifolia* (8.3%). *Heeria argentea* had the lowest yield of 0.8%.

Four out of 15 crude acetone extracts (*Harpephyllum caffrum*, *Searsia undulata*, *Sclerocarya birrea* and *Protorhus longifolia*) had significant antimycobacterial activity with minimum inhibitory concentration (MIC) values varying from 50-100 µg/mL. *Searsia undulata* had the highest activity against most mycobacteria, followed by *P. longifolia*. *Mycobacterium fortuitum* was a reasonably good predictor of activity against MDR-TB isolates (correlation coefficient =0.65). *Searsia undulata* extracts had significant antimycobacterial activity with the lowest MIC value of 70 µg/mL for *M. aurum* and *M. fortuitum* followed by *M. smegmatis* with MIC = 90 µg/mL. *Protorhus longifolia* also had significant antimycobacterial activity against all three non-tuberculous mycobacteria with MIC values of 110 µg/mL for both *M. aurum* and *M. fortuitum* and 70 µg/mL for *M. smegmatis*. *Searsia lancea*, *S. birrea* and *H. caffrum* had moderate activity of MIC = 420, 520 and 590 µg/mL against *M. aurum,* respectively, whereas the same plant species (*S. lancea*, *H. caffrum* and *S. birrea*) had moderate activity with low MIC values of 110 to 210 µg/mL for *M. smegmatis* and *M. fortuitum,* respectively. *Searsia undulata* had significant activity against all mycobacteria including MDR - *M. bovis* and *M. tuberculosis* with MIC values ranging from 50 to 110 µg/mL and the lowest MIC of 50 µg/mL against *M. tuberculosis* ATCC strain H37Ra.

Bioautography using *M. aurum* and *M. fortuitum* worked well as indicators of the R_f values of active compounds yielding strong zones of inhibition. The leaf extracts of *S. undulata* and *P. longifolia* had more than 10 different antimycobacterial compounds, whereas the other plant species showed none or only one to two active compounds depending on the non-tuberculous *Mycobacterium* spp. involved.

Based on good antimycobacterial activity and low cytotoxicity, *Searsia undulata* was further processed to isolate the active antimycobacterial compounds. The amount of 1.5 kg of dried fine powder of *S. undulata* leaves was processed for acetone bulk extraction, followed by solvent to solvent fractionation and separation by silica gel column chromatography. The isolation was bioassay-guided; fractions collected at different stages were tested using two-fold serial dilution to determine the minimum inhibitory concentration using the previous isolates of pathogenic MDR - *M. bovis*, *M. tuberculosis* and rapidly growing mycobacteria, *M. aurum*, *M. fortuitum* and *M. smegmatis*. The active compounds, visible on biautography, were targeted during the bioassay-guided isolation using thin layer chromatography as fingerprints to locate those active compounds.

Solvent to solvent fractionation yielded five fractions, namely chloroform, hexane, butanol, 35% H_2O-methanol and water fraction. Chloroform, butanol and 35% H_2O-methanol fractions had clear zones of inhibition on biautography whereas the water fraction did not show any clear zones of activity.

The chloroform fraction was further separated and yielded 55 fractions that were combined into 12 fractions (F1-F12), based on similar patterns on TLC fingerprints. Three out of the 12 fractions showed similar compounds using bioautography. These fractions were tested as already mentioned above under "quantitative antimycobacterial of fractions" and F5-F7 had good MIC values. These three semi-purified fractions were combined and separated further.

Fractions and compounds collected during different stages of isolation had good antimycobacterial activity. Some of the main fractions obtained during solvent-solvent fractionation, namely chloroform, butanol and 35% H_2O methanol, showed similar compounds inhibiting the growth of non-tuberculous *Mycobacterium* spp. on bioautography although TLC fingerprints of the butanol fraction did not show visible compounds. The chloroform fraction revealed the presence of several potential active fractions on the bioautogram plate sprayed with *M. aurum* whereas the plate sprayed with *M. fortuitum* had fewer active fractions which corresponded to a lower total activity observed with the combined sub-fractions F5-F7 derived from the chloroform fraction. The variation of activity observed on bioautography could be explained by the different sensitivity of mycobacterial species towards the potential antimycobacterial phytochemicals present in the fractions.

Sub-fractions F1-F12 derived from the chloroform fraction had MIC values from 58 to 468 µg/mL. Sub-fractions F5, F6 and F7 had significant antimycobacterial activity and were selected for further fractionation towards isolation of compounds with MIC values of 117 µg/mL for *M. aurum*, 234 µg/mL for *M. fortuitum* and 58 µg/mL for *M. smegmatis* for all fractions except F7 with MIC value of 117 µg/mL against *M. smegmatis*. Fractions F5 and F6 showed the lowest value of 58 µg/mL against *M. smegmatis*. Fractions F5, F6 and F7 were active against all three non-tuberculous

Mycobacterium spp. The total activity of each fraction (Mass/MIC) was calculated as this indicates which fraction could be the best candidate for organic production of the compound. Among all the fractions, F3 showed the highest total activity of 19 765 mL/g on its own for *M. aurum* and less for *M. fortuitum* and *M. smegmatis* whereas F5, F6 and F7 had a combined total activity of 44 181 mL/g against *M. smegmatis* with F6 and F5 showing the highest activity of 19 793 and 17 154 mL/g, respectively, against *M. smegmatis.* F7 had the highest activity of 7 262 mL/g for both *M. aurum* and *M. smegmatis* and 3 627 mL/g for *M. fortuitum*. The total activity of the combined fractions F5-F7 was the highest with *M. smegmatis* followed by total combined activity of 25 716 mL/g with *M. aurum.*

Three compounds were isolated from the leaves of *S. undulata* and these had good antimycobacterial activity against rapidly growing *Mycobacterium* spp*.* with MIC values ranging from 23.44 to 250 µg/mL. Two of the compounds (SLN1 and PK-B) had MIC values of 23.44 µg/mL, the lowest value for *M. fortuitum*, and MIC = 31.25 µg/mL for *M. aurum* and *M. smegmatis.* In addition, compound 3, had activity against all three mycobacteria with MIC = 32.25 µg/mL against *M. aurum* and *M. fortuitum* with the highest value of 46.88 µg/mL against *M. smegmatis*.

Two of the isolated compounds were subjected to MS and NMR spectroscopy. The first compound (SLN1) was identified as betulonic acid. The second compound (PK-B) appears to be a novel compound as it did not match with any published records using Scifinder search tool. This compound will be further identified using crystallization, melting point determination and Fourier Transform Infrared (FTIR) spectroscopy. This is the first report of isolation of betulonic acid from *S. undulata*. This compound has been reported to have anti-proliferative (anticancer) activity which could relate to the high cytotoxicity of the crude extract observed on human liver cancer cells. Other properties reported include antiviral, anti-inflammatory and antimicrobial. Besides the above three compounds, the isolation of the other active compounds is still underway, forming the research focus subsequent to this PhD study.

Cytotoxicity of the 15 acetone crude extracts and compounds of *S. undulata*

Cytotoxicity was assessed using a tetrazolium colorimetric assay (MTT) against Vero monkey kidney, human hepatoma (C3A) and murine macrophage (RAW 264.7) cell lines. Plants extracts of *H. caffrum*, *L. discolor*, *P. longifolia*, *S. undulata* and *S. birrea* with good antimycobacterial activity (significant to moderate) and showing one or more visible zones of inhibition on bioautograms were selected for cytotoxicity testing. All crude extracts from the above-mentioned plant species had low cytotoxicity against the three cell lines except *S. undulata* which had high toxicity against C3A cells and relatively low cytotoxicity on Vero kidney cells. This finding deserves further investigation as it may indicate that *S. undulata* extracts have good anticancer activity. *Searsia undulata* had moderate cytotoxicity with LC_{50} = 34 µg/mL for C3A and LC_{50} of 50 and 120 µg/mL for Vero kidney and RAW cells respectively with good selectivity indexes of 7.08 on Vero and more than 10 on RAW cells for non-tuberculous and MDR-TB mycobacteria. *Protorhus longifolia* had LC_{50} of 620 µg/mL on C3A cells, 880 µg/mL on Vero cells and more than 1 000 µg/mL on RAW cells with the highest selectivity index on Vero cells of 12.6 for *M. smegmatis* followed by 8.02 for *M. aurum*, *M. fortuitum* and MDR-TB. Extracts with SI > 1 may indicate better safety as they are less toxic to the mammalian cells but more toxic to pathogens but this will need to be confirmed with *in vivo* tests.

Only three compounds out of the five which were isolated in sufficient quantity were tested for cytotoxicity using Vero cells. PK-B was the least toxic with LC_{50} of 210 µg/mL whereas compound 3 and SLN1 showed moderate cytotoxicity of 31.3 µg/mL and 47.7 µg/mL, respectively.

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LIST OF ABBREVIATIONS AND SYMBOLS

Ac	Acetone
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BCG	Bacille Calmette Guerin (*M. bovis* attenuated vaccine strain)
BEA	Benzene/ethanol/ammonia (18:2:0.2)
BI	Bayesian inference
BLAST	Basic alignment search tool
C	Carbon
C3A	Human liver hepatoma cells
^{13}CNMR	Carbon 13 NMR
CEF	Chloroform/ethyl acetate/formic acid (10:8:2)
CFU	Colony forming units
$CHCL_3$	Chloroform
CP	Compound
1D	One dimensional
2D	Two dimensional
DAFF	Department of Agriculture, Forestry and Fisheries
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagles' Medium
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DR	Direct repeat
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme-linked immuno-sorbent assay
EMB	Ethambutol
EMEM	Minimal essential medium eagle with L- glutamine
EMW	Ethyl acetate/methanol/water (10:1.35:1)
EtOAc	Ethyl acetate
FAO	Food and Agriculture Organization
FBS	Foetal bovine serum
FTIR	Fourier transform infra-red spectroscopy

GC-MS	Gas chromatography-mass spectrometry
^{1}HNMR	Proton NMR
H37Ra	*Mycobacterium tuberculosis* attenuated strain
Ha	*Harpehyllum caffrum*
HCl	Hydrochloric acid
HEX	Hexane
HIV	Human immunodeficiency virus
H_2O	Water
IFN	Interferon
IGRA	Interferon - gamma release blood assays
INH	Isoniazid
IL	Interleukin
INT	p-Iodonitrotetrazolium
LAM	Lipoarabinomannan
LC_{50}	50% Lethal concentration
LC-MS	Liquid chromatography mass spectrometry
LJ	Löwenstein - Jensen
M7H9	Middlebrook 7H9
M7H10	Middlebrook 7H10
MA	*Mycobacterium aurum*
MAC	*Mycobacterium avium* complex
MAFFT	Multiple alignments by fast Fourier transformation
MALDI – TOF	Matrix assisted laser desorption ionization time of flight
MB	*Mycobacterium bovis*
MDR	Multidrug-resistant
MEGA 5	Molecular evolutionary genetic analysis
MEM	Minimum Essential Medium
MeOH	Methanol
MF	*Mycobacterium fortuitum*
MIC	Minimum inhibitory concentration
MIRUs	Mycobacterial interspersed repetitive units
MIT	Multi - inter - transdisciplinary
MGIT	Mycobacteria growth indicator tube
MH	Mueller Hinton

ML	Maximum likelihood
MOTT	Mycobacteria other than tuberculosis
MP	Maximum parsimony
MS	*Mycobacterium smegmatis*
MSH	Mycothiol
MTB	*Mycobacterium tuberculosis* complex
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide
NALC – NaOH	N - acetyl - L - cystein - sodium
NCTC	National Collection Type Cultures
NHLS	National Health Laboratory Service
NJ	Neighbor - joining
NMR	Nuclear magnetic resonance
NTM	Non - tuberculous mycobacteria
OADC	Oleate albumin dextrose catalase
OD	Optical density
OIE	Office International des Epizooties
OP	Onderstepoort
p	probability
PA	Pyrazinamide
PACT	Polymyxin B, amphotericin B, carbenicillin and trimethoprim
PAMPs	Pathogen - associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PL	*Protorhus longifolia*
PPEM	Potentially pathogenic environmental mycobacteria
PPD	Purified protein derivative
PPM	Potentially pathogenic mycobacteria
PRRs	Pattern recognition receptors
r	Pearson's correlation coefficient
RAPD	Random amplified polymorphic DNA
RAW 264.7	Murine macrophage cells
RCBH	Reverse cross blot hybridisation
RIDOM	Ribosomal differentiation of medical microorganisms
RFLP	Restriction fragment length polymorphism

RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SANBI	South African National Botanical Institute
SI	Selectivity index
R_f	Retardation factor or retention factor
RIF	Rifampin
TA	Total activity
TB	Tuberculosis
TBST	Tris buffered saline with Tween
TLC	Thin layer chromatography
TMB	3,3,5,5 – Tetra methyl - benzidine
TNF	Tumour necrosis factor
VNTR	Variable number of tandem repeat
WHO	World Health Organization
XDR	Extensively drug resistant
ZN	Ziehl - Neelsen

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LIST OF PUBLICATIONS

Research articles already published from this thesis:

Kabongo-Kayoka PN, Obi CL, Nakajima C, Suzuki Y, Hattori T, Eloff JN, Wright J, Mbelle N and McGaw LJ. 2015. Novel *Mycobacterium avium* complex isolated from Black Wildebeest (*Connochaetes gnou*) in South Africa. *Transboundary Emerging Diseases*. Doi: 10.1111/TBED.12460

Kabongo-Kayoka PN, Obi CL, Eloff JN and McGaw LJ. 2016. Antimycobacterial activity and low cytotoxicity of leaf extracts of some Anacardiaceae tree species. *Phytotherapy Research*. Doi: 10.1111/PTR-0292

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Conference presentations from the thesis:

Local conferences

- **Walter Sisulu University (WSU) International Research Conference. 2010.** Isolation, antibiogram profile and molecular characterization of *Mycobacterium* species from cattle and black wildebeest in Mpumalanga Province. WSU, Eastern Cape, 18-20th August 2010, an oral presentation.

- **World Veterinary Congress. 2011.** New *Mycobacterium* species isolated from Black Wildebeest, South Africa. World Vet Congress, Cape Town Conventional Centre, 10-14th October 2011, an oral presentation.

- **Faculty Day. 2014.** Leaf extract of selected Anacardiaceae trees have excellent antimycobacterial activity. Faculty of Veterinary Science, University of Pretoria, 4th September 2014, an oral presentation.

- **South African Association of Botanists, 41^St^ Annual Conference**. **2015.** Fractions of *Searsia undulata* (Anacardiaceae) have excellent antimycobacterial activity. Tshipise Forever Resort, Limpopo, 11-15th January 2015, an oral presentation.

International conferences:

- **International Infectious Diseases Conference**. **2010.** Isolation and molecular characterization of *M. avium*, *M. bovis* and *M. tuberculosis* in this era of HIV/AIDS in South Africa. University of Tohoku, Japan, Sendai, 13-16th January 2010, oral presentation.
- **International Journal of Arts and Sciences Conference. 2016.** High antimycobacterial activity and low cytotoxicity of *Searsia undulata* (Anacardiaceae) - An Overview. University of London, United Kingdom 8-11th November 2016, an oral presentation.

Papers prepared for publication:

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Kabongo-Kayoka PN, Obi CL, Eloff JN and McGaw LJ. 2017. Isolation of a novel antimycobacterial compound from *Searsia undulata. Planta Medica*

Kabongo-Kayoka PN, Obi CL, Eloff JN and McGaw LJ. 2017. Antioxidant, anti-inflammatory and inhibition of nitric oxide production of the potential antimycobacterial plants, *Protorhus longifolia* and *Searsia undulata. BMC Complementary and Alternative Medicine*

CHAPTER 1:
GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Many human diseases that are new, emerging or re-emerging at present, are caused by pathogens that originate from animals or products of animal origin (Müller *et al*., 2013). Tuberculosis can be foodborne or airborne and is one of the most important human infections in the world with more than 2 million new cases occurring each year, mostly in developing countries (WHO, 2015).

The emergence of multi-drug resistant (MDR) strains of *Mycobacterium tuberculosis* associated with human immunodeficiency virus (HIV) / acquired immunodeficiency syndrome (AIDS) is of great epidemiological concern. Species belonging to the *Mycobacterium tuberculosis* (MTB) complex include *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canetti*, *M. pinnipedii*, *M. caprae*, *M. microti*, *M. mungi*, *Dassie bacillus*, *Oryx bacillus (M. orygis)* and *M. surricatae* (Alexander *et al*., 2010; Azé *et al*., 2015; Clarke *et al*., 2016; Helden *et al*., 2009). There are other pathogenic non-tuberculous mycobacterium (NTM) species for example *Mycobacterium avium* complex (MAC) which consists of two closely related species, *M. avium* and *M. intracellulare* (Legrand *et al*., 2000). The importance of NTM has received much attention during the past decade, especially in humans. They are found widely in soil, water, near human settlements and aerosols and can be associated with colonization, serious infection or pseudo-outbreaks with a wide variety of presentations (Biet *et al*., 2005; Kankya *et al*., 2011; Katale *et al*., 2014).

The isolation of NTM from human clinical samples of patients (HIV positive or HIV negative) with pulmonary symptoms as suspected cases of tuberculosis has increased over the years and has been observed in different countries in Africa, America and Europe (Kankya *et al*., 2011; Katale *et al*., 2014; Mirsaeidi *et al*., 2014a; Moore *et al*., 2010). In animals, the clinical significance of NTM has yet to be elucidated in the disease-causing process (Chege *et al*., 2008; Kankya *et al.,* 2011; Katale *et al*., 2014).

The management, treatment and infection control measures differ significantly between *M. tuberculosis* and NTM infections. One hundred and sixty species of NTM have been reported worldwide, of which more than 60% are pathogenic to animals or humans (BoRam *et al*., 2014; Tortoli, 2014). In South Africa, reports on the isolation of NTM in animals, humans and the environment and their effects in disease-causing processes are limited (Gcebe *et al*., 2013; Kabongo-Kayoka *et al*., 2015; Michel *et al*., 2007; Müller *et al*., 2011).

As far as tuberculosis is concerned in humans, there are several underlying medical conditions that are associated with an increased risk of progressing to tuberculosis such as HIV infection, diabetes mellitus, renal failure, malnutrition or advanced malignancy but the disease can develop in people who do not have any of the mentioned risk factors, most likely due to genetic susceptibility (Gopathi *et al*., 2015).

South Africa has one of the highest tuberculosis incidence rates in the world (WHO, 2015). Twenty one million people are infected with TB of which 5-10% may develop active tuberculosis whereas the majority of individuals (90-95%) infected with *M. tuberculosis* will never develop the disease (Lienhardt *et al*., 2016). The clinical manifestation of tuberculosis is worsened when associated with HIV/AIDS due to the concomitant immunosuppression (Swaminathan and Chandrasekaran, 2014).

The most common route of infection (95%) is inhalation of infectious droplet nuclei through the air-borne route but the foodborne/alimentary route also plays an important role in the dissemination of the disease as does the handling of contaminated fomites (Inderlied, 2004; Wani, 2013). The exposure to *M. tuberculosis* bacilli does not always lead to infection and not all the patients are infectious. Only individuals with active pulmonary or laryngeal tuberculosis are infectious. Infectious individuals propel TB bacilli in the air by coughing, sneezing, spitting, talking or singing. The risk of infection is directly related to the number and distribution of tubercle bacilli in the inhaled and respired air (Inderlied, 2004). Coughing and talking for 5 minutes can produce 3 000 infectious droplet nuclei and sneezing can produce over a million particles (Alland *et al*., 1994). Inhaled droplet nuclei that are less than 100 nm in diameter usually travel through the airway until they reach the alveoli whereas the larger particles that are deposited on the way are removed through the normal mechanism of airway clearance (Dannenberg Jr, 1989). The first step of possible infection is the contact between the bacteria and lung alveolar epithelial mucosal cells and attending macrophages. The inhaled bacilli are phagocytosed, processed, and presented by alveolar macrophages to the T-lymphocytes (Gopathi *et al*., 2015). The probability of an infection depends on the ability of the mycobacterium to survive within the macrophages, in this way evading many host defence mechanisms (Figure 1.1). During this phase, unless a patient receives prophylactic treatment, symptomatic disease will eventually occur in 5-10% of infected patients (Inderlied, 2004; Lin and Flynn, 2010).

Several methods have been described for identifying *Mycobacterium* species. Conventional methods continue to be used such as smears, mycobacterial cultures and chest radiography but other technologies have emerged that include nucleic acid amplification tests, immune based assays, skin path test and rapid culture systems (Barnard *et al*., 2008; Pai *et al*., 2006). Mycobacterial cultures and histopathology are essential for confirmation of TB infection; molecular characterization is used for identification and studying the spatial spread of mycobacteria whereas phylogenetic analysis has been used for analysis of morphological and molecular data to define relationships of mycobacteria isolates (Barnard *et al*., 2008; Pai *et al*., 2006).

Control measures of TB in animals depend on the primary objectives for the specific ecosystem, being wild animals or domestic animals. Currently, control and monitoring of infected animals are done using the intradermal tuberculin test. In humans, vaccination remains the ultimate control measure but it has been reported that the vaccination is no longer as effective as it was in the past. There are 15 vaccines that are currently on trial in different countries (WHO, 2015).

In general, TB is not treated in animals - the testing and slaughter policy is followed whereas in humans, the treatment of tuberculosis has become a challenge due to emerging multi-drug resistant strains (MDR). The molecular basis of MDR tuberculosis has been well documented (Bosne-David *et al*., 2000; Cingolani *et al*., 1999; Musser, 1995; Sreevatsan *et al*., 1997). Mutations that occur in the target genes of *M. tuberculosis* and *M. bovis* strains are the most common mechanisms of resistance encountered for primary antimycobacterial agents such as rifampin, isoniazid and streptomycin. The World Health Organization estimates that up to 50 million persons worldwide may be infected with drug resistant strains of *M.*

tuberculosis (WHO, 2015). As far as genetic assessment is concerned, there are limited data available on drug resistant *M. bovis* strains and this species is transmissible to humans (Blazquez *et al*., 1997).

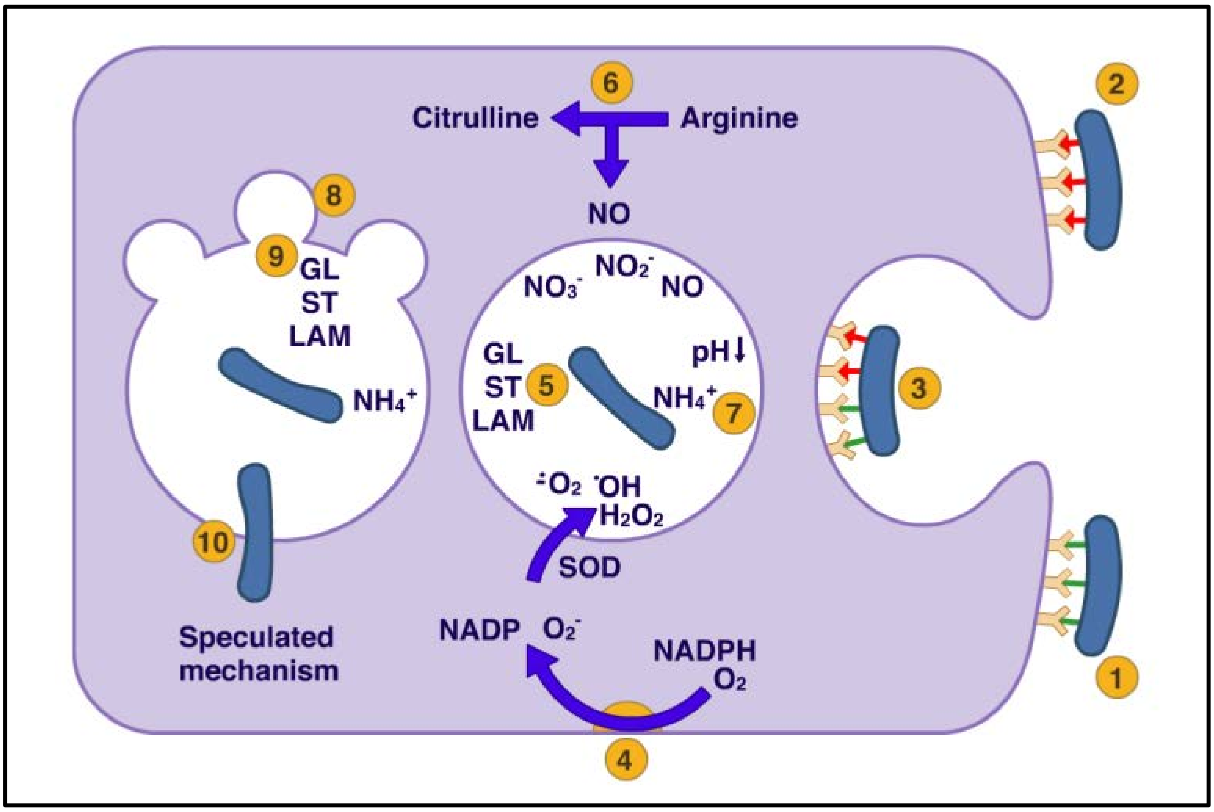


Figure 1.1: Macrophage phacocytosis and evasion of tubercle bacilli (Inderlied, 2004)

(1). The tubercle bacilli bind via lipoarabinomannan (LAM) or complement receptors (2); phagocytosis occurs (3) with the activation of an oxidative burst with superoxide dismutase (SOD) (4), glycolipids (GL), sulfatides (ST), thiols and LAM downregulate the oxidative burst (5), reactive nitrogen intermediates may play a role in antimycobacterial activity (6), similar role is played by the acidic pH of the phagolysosome (7). The production of ammonia by tubercles may diminish the effect of reactive nitrogen intermediates (8) and contribute to the failure to form a phagolysosome fusion (9). The tubercle bacilli may evade the antimycobacterial activities of the phagolysosome by producing a hemolysin that releases the bacilli into the cytoplasm (10).

Plants have been used as resources for many traditional medicine systems throughout the world for centuries and continue to provide people with new remedies. Numerous useful drugs were developed from compounds isolated from medicinal plants. Up to this day, this strategy remains an essential route to new pharmaceuticals (Balunas and Kinghorn, 2005; Jachak and Saklani, 2007). There are also few publications showing activity of certain plants against the rapidly growing mycobacteria, namely *M. aurum, M. bovis* BCG*, M. smegmatis* and *M. fortuitum* (Chimponda and Mukanganyama, 2010; Ghaemi *et al*., 2011; Mariita *et al*., 2010; Nguta *et al*., 2016). Further investigation is needed to evaluate activity against the pathogenic mycobacteria, namely *M. bovis* and *M. tuberculosis*.

A large proportion of the populations in developing countries use traditional medicines for primary health care. It is estimated that 80% of the world's population utilize plants as their primary source of medicinal agents (Cowan, 1999; Fabricant and Farnsworth, 2001). In South Africa up to 60% of the population consult traditional healers as an alternative to or in addition to Western health care providers (Van Vuuren, 2008). The reliance on traditional medicine can be attributed to availability, accessibility, affordability, extensive traditional knowledge and expertise within communities (Gurib-Fakim, 2006).

Much therapeutics used daily in South Africa is still derived from plants. This presents a valuable resource for research into the development of new pharmaceutical drugs and there has been a growing interest in natural and traditional medicines as sources of new medicinal products of commercial value (Mahomoodally *et al*., 2010; Rybicki *et al*., 2012). Approximately 30 000 plant species of South Africa form a useful potential pool for the screening of new therapeutic compounds. Conventional medicine has been accepting the use of traditional medicine once scientifically validated (Mukherjee *et al*., 2010). Some traditional healers use the different parts of the whole plant depending on the disease (Street *et al*., 2008; Van Wyk, 2008a).

It is often preferable to screen leaves rather than roots or bark for potential biological activity as part of promoting sustainability of the environment. Traditional health practitioners predominantly use water as an extractant but it has been reported that some compounds including antimicrobials are not commonly extracted by water (Kotzé and Eloff, 2002). Among other organic solvents, acetone has proven not to be bactericidal at the concentration used in the bioassay (Eloff *et al*., 2007) and therefore, it is a solvent of choice as extractant in the investigation of antimicrobial activities of plant extracts including antimycobacterial activities (Eloff, 1998a).

Isolation and identification of compounds from plants have been explored using different research techniques, among others bioassay-guided fractionation, which involves repetitive fractionation of extracts in order to determine the biological activity of each fraction against a specific organism (Brusotti *et al*., 2014). This method is time-consuming and labour intensive but provides a rational means to isolate bioactive compounds from a complex mixture (Bero *et al*., 2011).

Other methods that have been used comprise thin layer chromatography (TLC), gas or liquid chromatography, mass spectrometry, open column chromatography on silica gel, nuclear magnetic resonance (NMR) spectroscopy and Fourier Transform Infra-Red (FTIR) spectroscopy (Harborne, 1998).

Nuclear magnetic resonance is the most frequently used technique by chemist and biochemists to identify the structure of organic molecules. Samples may be analysed with 1-dimensional proton and 2-dimensional carbon 13 NMR spectroscopy (Exarchou *et al*., 2003; Ward *et al*., 2007).

1.2 LITERATURE REVIEW

1.2.1 Tuberculosis epidemiology

1.2.1.1 Tuberculosis in humans

Tuberculosis (TB) is one of the major causes of illness and death in the world, especially in Asia and Africa (Figure 1.2). The incidence of this disease may still be under-reported in countries with limited laboratory skills. According to the World Health Organization report (2015), 9.6 million of TB cases occurred in 2014, 1.5 million people died of TB, and among these mortalities, 1.1 million were HIV negative and 0.4 million HIV positive. While life expectancy has been increasing for HIV/AIDS individuals due to antiretroviral drugs, the battle against TB is still a challenge.

Tuberculosis remains one of the world’s biggest threats alongside HIV as a leading cause of death. It has been reported that the ratio of patients under treatment to patients newly notified as having MDR-TB or rifampicin resistant TB was 90% globally (WHO, 2015). Only 50% of MDR-TB patients were successfully treated globally. An estimated 9.7% of people with MDR-TB have extensively drug resistant (XDR) TB (WHO, 2015). South Africa has the highest incidence rate of TB per capita of 500 per 100 000 population (Figure 1.2). South Africa is on the list of 30 high burden TB countries in the world according to the World Health Organization (2015). A large proportion (59%) of people living with co-infection of HIV and TB has been reported in South Africa (WHO, 2015).

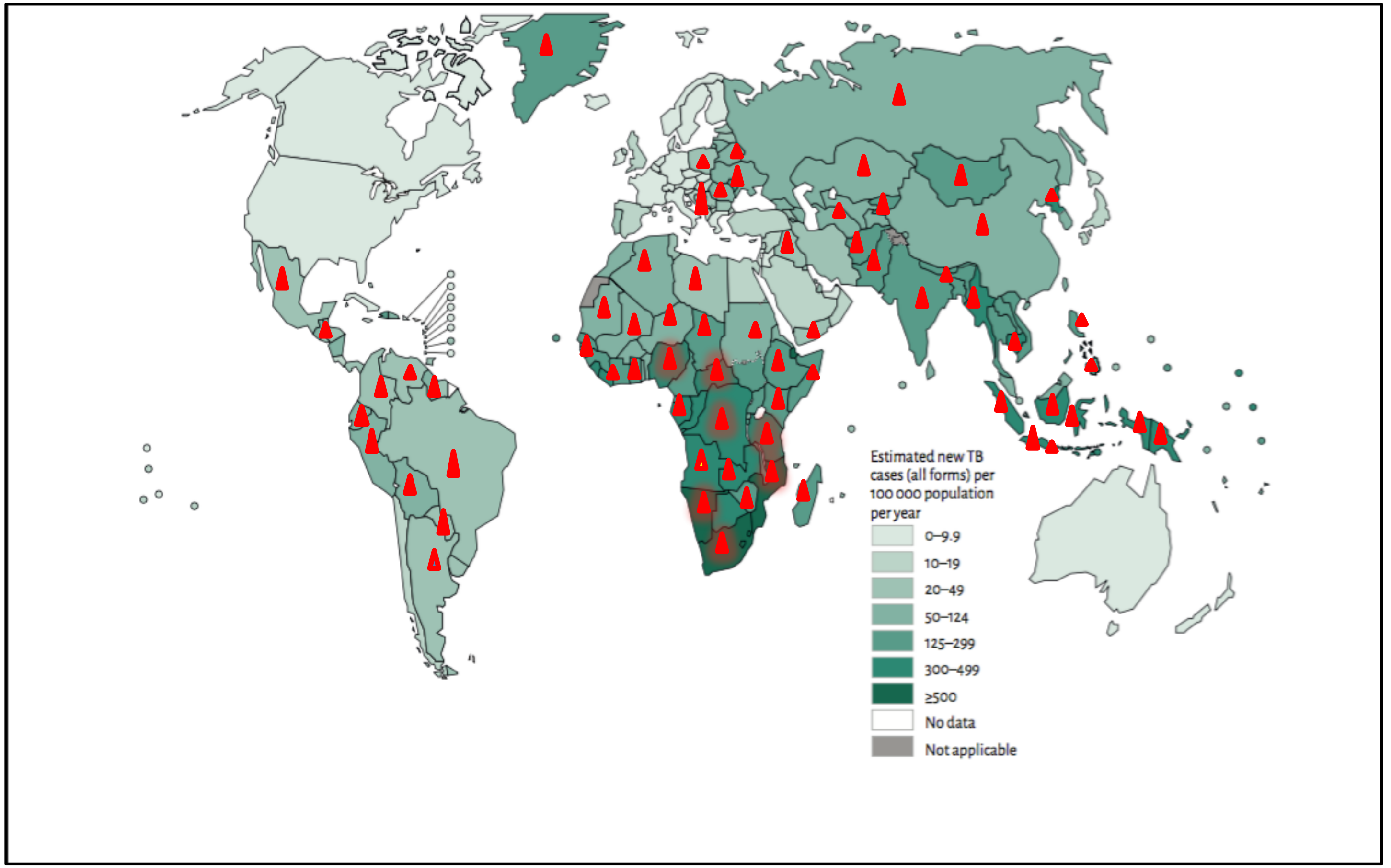


Figure 1.2: Map showing the estimated new TB cases in the world (WHO, 2015)

Source: courtesy of www.who.int

Most of the TB drugs currently in use were introduced more than 50 years ago. New drugs approved for treatment of TB, namely rifabutin and rifapentine, are not yet widely available (León-Díaz *et al*., 2013; Svensson *et al*., 2014). After a pilot trial conducted in South Africa (Diacon *et al*., 2012), a treatment programme was introduced in March 2014 for XDR-TB patients using the first new drug after more than 50 years, bedaquiline, initially known as diarylquinolone TMC 207. This drug has a new mechanism of action which consists of inhibiting ATP synthase (Ganihigama *et al*., 2015). Another new MDR-TB treatment using delamanid has also been tested (Xavier and Lakshmanan, 2014). Linezolid, an antibiotic discovered during the late 1990's, has been used in combination with bedaquiline (Sotgiu *et al*., 2013; Zumla *et al*., 2013). Antibiotic resistance is a continuous threat; therefore, research for alternative drugs is ongoing to broaden the range of effective and safe potential antimycobacterial drugs. Indeed, studies conducted in countries such as Europe, Africa, America and Canada have led to isolation of different chemical compounds, namely allicin, alkaloids, arjunic acid, anthraquinone, anthocyanidin, beta-sitosterol, benzophenanthridine alkaloids, crinine, decarine, mellagitannin, neolignans, elligitannin, ellagic acids, friedelin, gallic acid, galanthimine, glucopyranosides, glycosides, hydroxybenzoic acids, iridoids, phenylpropanoids, 1-epicatechol, leucopelargonidol, punicalagin, taraxerol and termilignan B (Chinsembu, 2016; Mukanganyama *et al*., 2015). Some of these compounds may provide leads to the development of new and less toxic drugs to lessen the global burden of TB and drug resistant *M. tuberculosis* strains.

1.2.1.2 Tuberculosis in animals

Tuberculosis in cattle is caused mainly by *M. bovis* and cattle are considered the true host; in other words they are the main maintenance host of *M. bovis* in domestic animals. In South Africa, *M. bovis* infection has been confirmed in the following wild species: African buffalo (*Syncerus caffer*), Greater Kudu (*Tragelaphus strepsiceros*), Lion (*Panthera leo*), Eland (*Taurotragus oryx*), Warthog (*Phacochoerus aethiopicus*), Bushpig (*Potamochoerus porcus*), Large spotted genet (*Genetta tigrina*), Leopard (*Panthera pardus*), Spotted hyena (*Crocuta crocuta*), Cheetah (*Acinonyx jubatus*), Chacma baboon (*Papio ursinus*), Impala (*Aepyceros melampus*) and Honey badger (*Mellivora capensis*) (Bengis *et al*., 2002, 1996; Hang'ombe *et al*., 2012; Hlokwe *et al*., 2013; Palmer *et al*., 2012). *Mycobacterium bovis* has also been isolated from bison, sheep, goats, equines, camels, pigs, wild boars, deer, antelopes, dogs, cats, foxes, mink, badgers, ferrets, rats, primates, llamas, elands, tapirs, elks, elephants, sitatungas, oryxes, addaxes, rhinoceroses, possums, ground squirrels, otters, seals, hares, moles, racoons, coyotes and several predatory felines including tigers and lynx (O'Brien *et al*., 2002 and OIE, 2014).

Tuberculosis is a zoonotic disease as it occurs in both humans and animals with similar clinical features (Cousins, 2001; De la Rua-Domenech, 2006a; Grange, 2001). It is primarily a respiratory disease and transmission of infection is mainly by the airborne route although the food-borne route is also encountered. It can also be systemic and affect different organs in the body (De la Rua-Domenech, 2006; Fanning, 1999).

Many susceptible species including man are considered spill-over hosts as they harbour *Mycobacterium bovis* as long as the maintenance host is present but still can spread the disease. In some instances, spill overs can become maintenance hosts and spill back to the initial hosts. The African buffalo (*Syncerus caffer*) in South Africa is considered a maintenance host for *M. bovis* (Bengis *et al*., 2002; Michel *et al*., 2010; Vos *et al*., 2001) while there is spill-over to other species (Vos *et al*., 2001). In Canada, the wapiti (*Cervus elaphus*) and bison (*Bison bison*) are reservoirs (Nishi *et al*., 2006); in USA, the white-tailed deer population is the wildlife reservoir for *M. bovis* (de Lisle *et al*., 2002; O'Brien *et al*., 2002; Schmitt *et al*., 2002); European badger (*Meles meles*) are reservoir hosts in Ireland, United Kingdom (Corner, 2006); brushtail possums (*Trichosurus vulpecula*) are the primary maintenance host in New Zealand (Nugent, 2011). Tuberculosis in captive deer or wild cervids has been observed in Europe and America; wild boars (*Sus scrofa*) are maintenance hosts for other wildlife and domestic animals in Europe (Naranjo *et al*., 2008). Wildlife may contaminate cattle by direct or indirect contact at the wildlife-livestock interface (Michel *et al*., 2010).

In South Africa, recent studies have also revealed that interspecies spreading of *M. bovis* is extending to different animal species in different provinces; spoligotyping has revealed that some strains are from the same origin which implies that there is indeed inter- and intra-species spreading of the disease (Hlokwe *et al*., 2014). This situation is of concern as it makes the control of TB difficult. In this study, adding to the animal species mentioned in the first paragraph, *M. bovis* was also isolated from cattle (*Bos taurus*), bushbuck (*Tragelaphus scriptus* and *Tragelaphus sylvaticus*) nyala (*Tragelaphus angasii*), waterbuck (*Kobus ellipsiprymnus*) and wildebeest (*Connochaetes gnou* and *Connochaetes taurinus*) (Hlokwe *et al*., 2014). Human infections with *M. bovis* have been reported (Cosivi *et al*., 1998; de Kantor *et al*., 2008; De Kantor *et al*., 2010; De la Rua-Domenech, 2006; Thoen *et al*., 2009). A study conducted in California confirmed cases of *M. bovis* in children and adults (Gallivan *et al*., 2015). *Mycobacterium bovis* in adults was associated with HIV or other immunosuppressive disease such as diabetes, whereas in children the clinical picture was usually mainly extra-pulmonary TB. Human to human transmission of *M. bovis* in immunocompetent patients has also been reported in France (Sunder *et al*., 2009).

Tuberculosis is not treated in animals; the test and slaughter approach is used to control the spread of the disease (Cousins, 2001). In South Africa, eradication of tuberculosis in livestock is sometimes compromised due to the lack of tuberculin stock (Matt Ekron, personal communication 2010). Adding to this, the implementation of the test and slaughter policy is either non-existent or compromised due to lack of farmers' compensation by the government subsidies and the increase of emerging farmers (Personal communication and observation during the course of this research 2009-2012; Department of Agriculture, Forestry and Fisheries; Musoke *et al*., 2015). In developing countries, human tuberculosis caused by *M. bovis* is probably under-reported as there are limitations of laboratories in identifying and differentiating *M. bovis* from *M. tuberculosis*. In countries where there is transmission of infection from endemically infected wildlife populations to cattle or other farmed animals, eradication is not feasible as wild animals play the role of maintenance hosts and control measures must be applied indefinitely, and this is also the case in South Africa (Keet *et al*., 2001; Michel *et al*., 2010).

Bovine TB has been reduced or eliminated in many developed countries but there are pockets of infected reservoirs in wildlife in Canada, the United Kingdom, the United States of America and New Zealand (Corner *et al*., 2009; de Lisle *et al*., 2002; Naranjo *et al*., 2008; Nugent, 2011). The prevalence of bovine TB has been found to be high in certain populations of animals. In South Africa, the highest prevalence of

70% was reported in buffaloes (Kriek *et al*., 1996) whereas in Zambia, the prevalence of 50% has been reported in Kafue where lechwe antelopes are the maintenance hosts (Munyeme *et al*., 2010). Infected wild animals are a source of infections for other animal species through ingestion of infected meat or carcasses by lions, cheetah and baboons. Sharing of extensive grazing land is also a factor in the spreading of the disease among animals (Munyeme *et al*., 2010). A similar situation has been reported in New Zealand where maintenance hosts are mainly ferrets (*Mustela furo*) and stoats (*Mustela erminea*); ferrets could also infect cattle and farmed deers.

The zoonotic transmission of TB infection in humans is likely to occur by inhalation of infected droplets which are expelled from the lungs during coughing of sick animals. Calves and humans can also become infected by ingesting raw milk from infected cows or by handling contaminated fomites on the farm or premises where infected animals are housed or post-mortem in the laboratory (Cosivi *et al*., 1998). China among others has been identified as a high burden TB country (WHO, 2015). It has been reported that cattle maintain both *M. bovis* and *M. tuberculosis*. Although *M. tuberculosis* was thought to be non-pathogenic to cattle, there has been evidence of *M. tuberculosis* isolated from cattle tissues with and without tuberculous lesions (Prasad *et al*., 2005; Une and Mori, 2007).

The course of the disease is slow; taking months or years to kill an infected animal, thus, an animal can spread the disease to many other animals in the herd before it begins to manifest clinical signs. Therefore, movement of undetected infected domestic animals and contact with infected wild animals are the major ways of spreading the disease (Cosivi *et al*., 1998; Hlokwe *et al*., 2014; Michel *et al*., 2008).

1.2.2 Mycobacteria

Bacterial ecology is broad and complex, therefore our knowledge is still limited as most bacteria are still unknown, with new enzymatic functions or new products to be isolated (Busse *et al*., 1996). Mycobacteria belong to the family Mycobacteriaceae and the genus *Mycobacterium*. The members of the genus *Mycobacterium* are genetically closer to each other than the microorganisms belonging to other genera, making identification a difficult and challenging task. This genus includes species that cause tuberculosis and those that cause disease conditions other than tuberculosis called “non-tuberculous mycobacteria” or NTM.

The genus *Mycobacterium* contains more than 170 species (http://www.bacterio.net/mycobacterium.html) most of which are classified as NTM or potentially pathogenic mycobacteria (PPM); (BoRam *et al*., 2014; Chege *et al*., 2008; Malama *et al*., 2014; Tortoli, 2014) and mycobacteria belonging to the *Mycobacterium tuberculosis* complex (MTC). The MTC comprises *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canetti*, *M. pinnipedii*, *M. caprae*, *M. microti*, *M. mungi*, *Dassie bacillus*, *Oryx bacillus (M. orygis), M. surricatae* and the attenuated *M. bovis* Bacille Calmette–Guerin (BCG) vaccine strain. With the exception of BCG, these species are obligate parasites, pathogenic and can cause tuberculosis (TB) in mammalian hosts (Alexander *et al*., 2010; Azé *et al*., 2015; Pittius *et al*., 2012; Vos *et al*., 2001). The prominent pathogens in the MTC include *M. tuberculosis* and *M. bovis*; both species are incriminated in cases of human and bovine tuberculosis because *M. bovis* is transmitted zoonotically.

Mycobacteria are aerobic, non-motile single cell, non-sporeforming and are straight or slightly curved rods. They are considered Gram-positive although they are not stained easily by Gram’s stain. They are grouped in the suprageneric rank of actinomycetes that, unusually, have a high content of guanine (61-71%) and cytosine (G+C) in the genomic deoxyribonucleic acid (DNA). The most prominent feature of mycobacteria that is uniformly present and distinctive to the genus is the complex, lipid-rich cell envelope (Figure 1.3). The structure of the mycobacterial cell envelope comprises a plasma membrane, a peptidoglycan layer, an arabinogalactan layer esterified to an uneven mycolate layer, and a glycolipid layer. Lipoarabinomannan and a small number of porins traverse the width of the mycobacterial envelope (Daffé and Reyrat, 2008). A common feature of the mycobacterial envelope is the lipid shell which is made of a thick, hydrophobic waxy cell wall rich in mycolic acids up to 50% (Chatterjee, 1997; Riley, 2006). This is one of the main characteristics of the genus responsible for acid fastness, extreme hydrophobicity, and resistance to injury such as desiccation and most disinfectants including the drugs (antibiotics) as well as distinctive immunological properties. It also contributes to the slow growth rate of some species by restricting the uptake of nutrients (Hett and Rubin, 2008; Niederweis, 2003). Based on the growth rate, mycobacteria are divided into two groups: rapid growers and slow growers. Rapid growers are separated from slowly growing mycobacteria according to the time required to produce clearly visible colonies which is about 7 days or less for rapid growers (Inderlied, 2004) whereas most pathogenic mycobacteria are slow growing organisms. *Mycobacterium smegmatis* (rapidly growing) has a generation time of 2-4 h whereas *M. tuberculosis* (slow grower) has a generation time of 16-18 h (Wayne, 1994). Adding to the lipids, peptidoglycan is another major component of the mycobacterial cell wall.

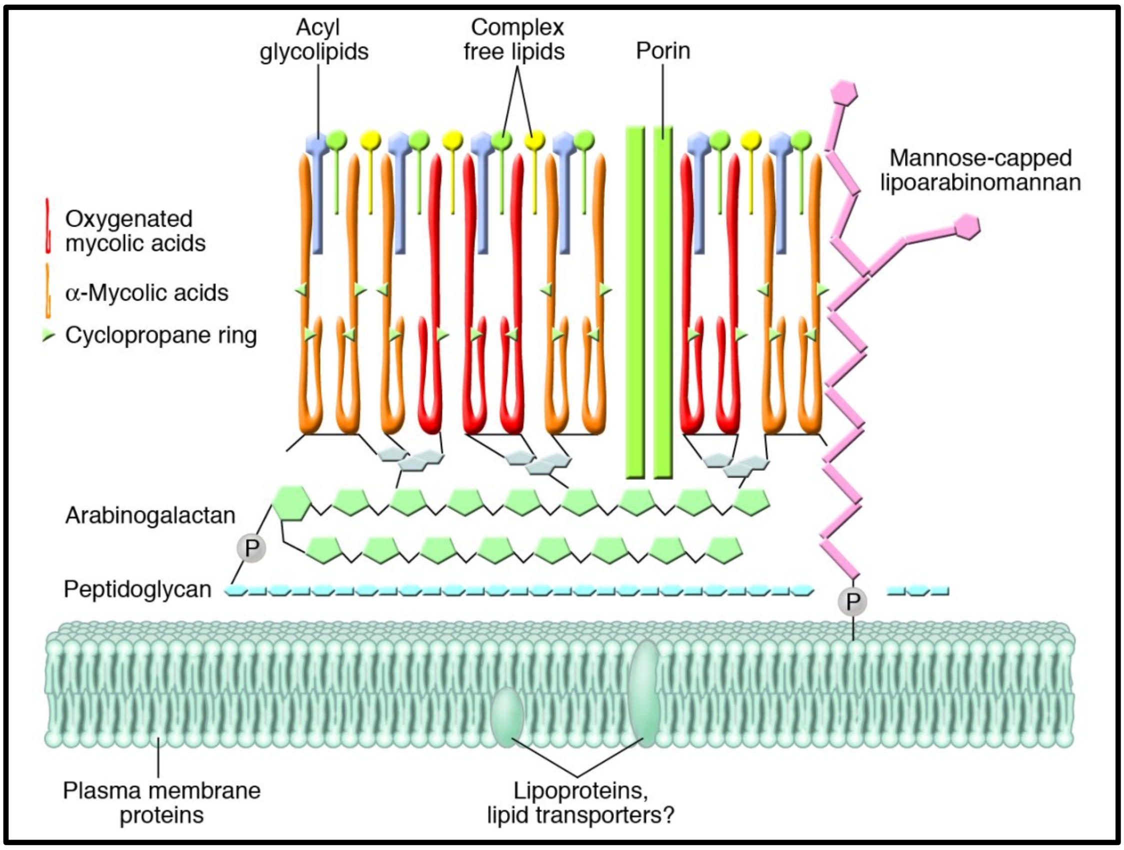


Figure 1.3: Mycobacterial cell envelopes (Riley, 2006)

Source: courtesy of www.jci.org

When processing samples for isolation, the resistance of the cell wall due to the high lipid content allows treatment of samples with acid or alkali to kill microbial contaminants without destroying the mycobacteria. Mycobacteria are acid-fast organisms. Ziehl-Neelsen is the stain of choice for their microscopic visualization; once stained with carbol fuchsin, they cannot be decolourized by acid-alcohol and hence they are referred to as “acid fast” (Figure 1.4). With Ziehl-Neelsen stain, the bacilli appear as stained dark pink rods against a deep sky-blue background. *Mycobacterium* and other closely related genera namely *Corynebacterium, Gordona, Tsukamurella, Nocardia, Rhodococcus* and *Dietzia* have similar cell wall compounds and structure and show some phenotypic resemblance.

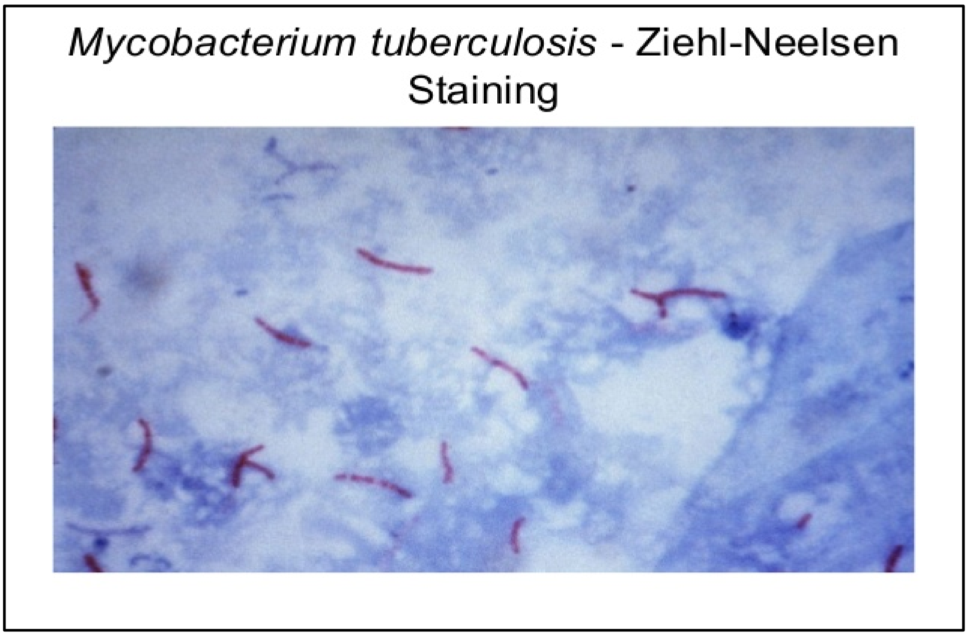


Figure 1.4: *Mycobacterium tuberculosis* stained by Ziehl-Neelsen method, appearing as dark pink straight and curved rods (Brehar, 2015)

Source: courtesy of www.slideshare.net

1.2.2.1 Mycobacterium tuberculosis

In the 1700s and early 1800s, tuberculosis prevalence reached its peak in Western Europe and the United States and was the most common cause of death. A hundred years later, it spread to Eastern Europe, Asia, Africa and South America (Bloom and Murray, 1992). Today, tuberculosis has re-emerged as one of the prevalent causes of death worldwide. *Mycobacterium tuberculosis* can also infect animals (Maslow and Mikota, 2015; Michel *et al*., 2010; Mikota *et al*., 2001). It is the main cause of human tuberculosis and was first described by Robert Koch in 1882. He was able to reproduce the disease in guinea pigs and rabbits and isolated the bacillus in pure culture (Mikota *et al*., 2001). This microorganism is also known as Koch’s bacillus. As previously mentioned, it belongs to the *M. tuberculosis* complex. In 1891, Koch developed tuberculin, a purified sterile protein extracted from culture of *M. tuberculosis*, which was used for immunity or treatment of the disease. It is still a valuable diagnostic and control tool for the detection of the disease caused by *M. tuberculosis* or *M. bovis*; it is used in cattle to identify infected animals that react positively on the tuberculin test. These animals are either slaughtered or isolated in control programmes. Purified protein obtained from cultures of *M. bovis* or *M. avium* can also be used in the comparative tuberculin test (Corner, 2007; Musoke *et al*., 2015; Tweddle and Livingstone, 1994).

Microscopically, *M. tuberculosis* appears as a fairly large, slender, non-motile, slightly curved or straight, rod shaped bacterium. The rods are 1.0 to 4.0 µm in length and 0.2 to 0.5 µm in width surrounded by a multi-layered cell wall of about 20 nm thicknesses. One of the features of this mycobacterium is formation of cords (Figure 1.5), which is due to the presence of a glycolipid loosely bound in the outer layer of the cell wall (Chan *et al*., 1991; Glickman *et al*., 2000). The cord factor is thought to be important as a virulent factor because it inhibits secretion of tumour necrosis factor alpha (TNF) by macrophages (Clay *et al*., 2008; Ryll *et al*., 2001).

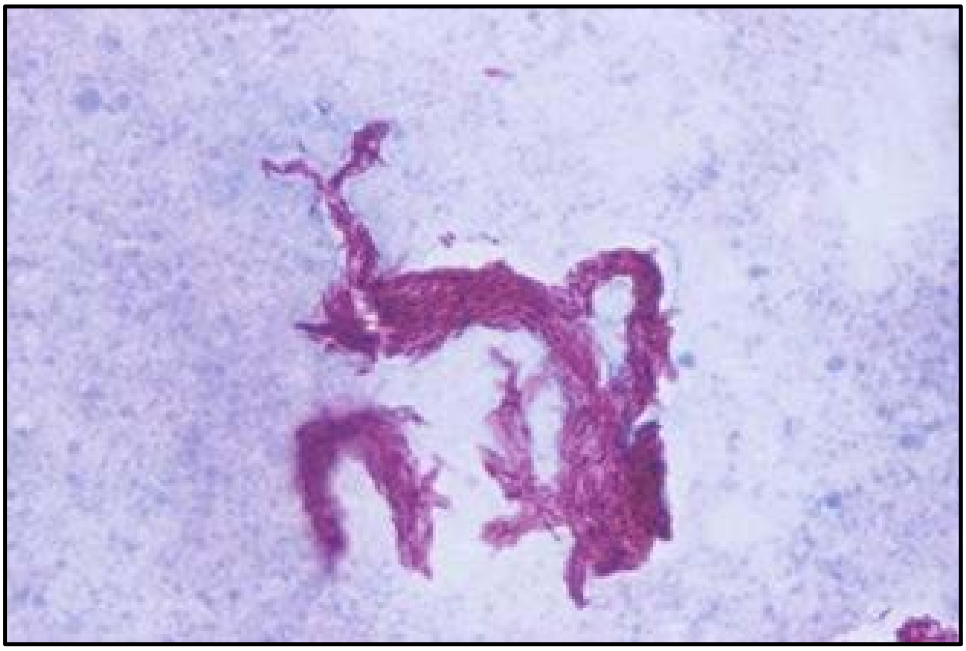


Figure 1.5: *Mycobacterium tuberculosis* cord factor.

Source: courtesy of www.intranet.tdmu.edu.ua

Macroscopically, *M. tuberculosis* grows on medium containing egg yolk (Löwenstein-Jensen) and colonies appear as raised, dry, cream-yellowish coloured colonies. The colonies are tough and difficult to pick up with a loop from the surface of the agar (Figure 1.6).

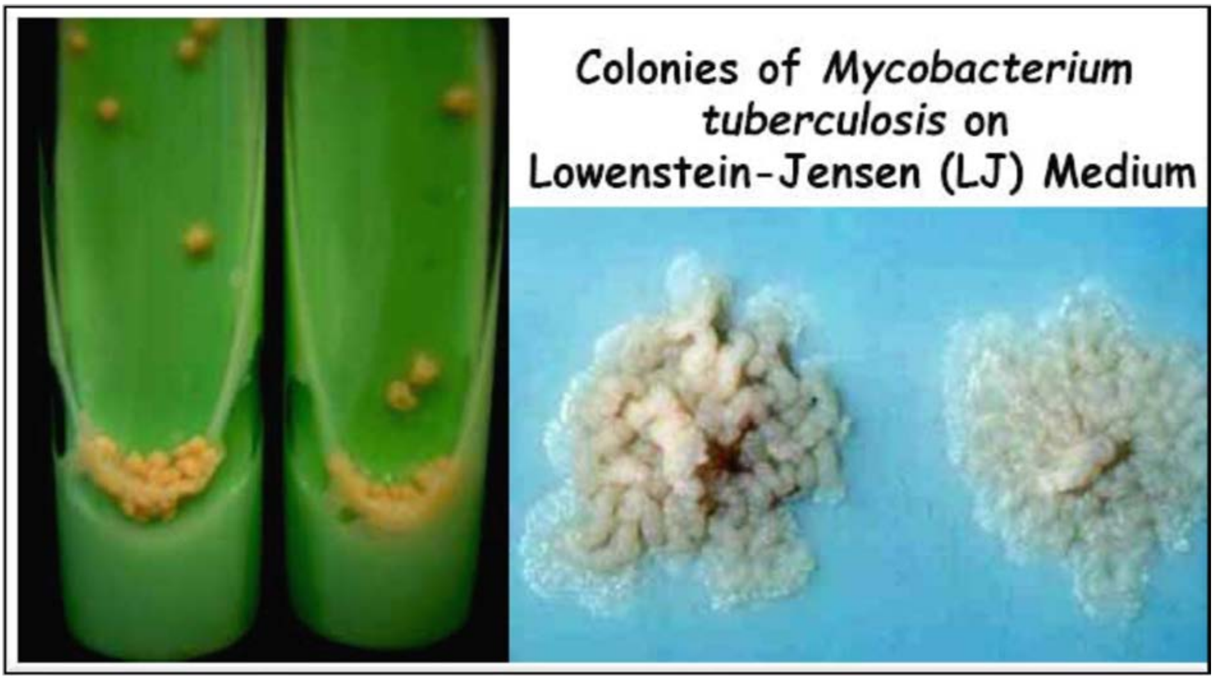


Figure 1.6: Colonies of *M. tuberculosis* on Löwenstein-Jensen agar slope tubes

Source: courtesy of www.microbiologyinfo.com

People with compromised immunity due to factors such as malnutrition, physical stress, poor access to health services, overcrowded conditions, and other diseases namely HIV/AIDS, diabetes etc. are more susceptible to TB. In general, the natural body defences of an individual can prevent the infection from developing into active disease. Besides human infection, *M. tuberculosis* and *M. tuberculosis*-like organisms have been reported in a wide range of species that include non-human primates, elephants and other exotic ungulates, carnivores, marine mammals and psittacine birds, and transmission from some of these species namely non-human primates, Asian elephants and psittacine birds to humans has been reported (Maslow and Mikota, 2015; Ocepek *et al*., 2005).

1.2.2.2 Mycobacterium bovis

Bovine tuberculosis existed in the Mediterranean littoral before classical times. From Northern Italy, it spread to Western Europe and Great Britain then, cattle imported from Holland and Great Britain spread the disease to other parts of the world that were colonized by those countries (Myers and Steele, 1969).

The first reference to animal tuberculosis in South Africa was made by Hutcheon in 1880 after importation of European breeds; in 1902, many animals imported from Australia, Argentina, Madagascar and England were destroyed in Durban after a positive reaction in the tuberculin test (Henning, 1956). The spread in South Africa was originally confined to dairy farms but more beef herds have been found to be infected (Hlokwe *et al*., 2011; Musoke *et al*., 2015). Bovine tuberculosis was first recorded in Zimbabwe in 1908 in a heifer imported from the Cape Peninsula. With movement of cattle and animals across the world, *Mycobacterium bovis* has since been isolated in domestic and wild animals and humans in different African countries (Ayele *et al*., 2004; Cosivi *et al*., 1998; Hlokwe *et al*., 2014; Marcotty *et al*., 2013; Michel *et al*., 2010; Munyeme *et al*., 2010a; Munyeme *et al*., 2010b; Musoke *et al*., 2015; Thoen *et al*., 2009).

Bovine tuberculosis has been a notifiable and controlled disease in South Africa since 1911. Under the Animal Diseases Act No 27 of 1903, individual animals and herds which had been in contact with cases that tested positive and with infected animals based on lesions observed at the abattoir were subjected to compulsory tuberculin testing. Reactors were slaughtered and their owners compensated. Later on, this act was replaced by voluntary accreditation of the herd meaning that farm owners voluntarily joined the bovine tuberculosis eradication scheme. The scheme objective of total eradication through a test and slaughter policy was introduced in 1969 and it has since been in operation to date under the Animal Diseases Act No 35 of 1984 (www.nda.agric.za).

Although *Mycobacterium bovis* is of greatest concern in cattle as the primary host, it can occur in humans and many other domesticated and wild animal species. Goats and swine are highly susceptible to infection, while sheep and horses are more resistant (Pavlik *et al*., 2003). Besides known maintenance reservoir hosts previously mentioned, some authors believe that there are species that could be spill-over hosts that maintain the organism only when its population density is high. Species reported to be spill-over hosts include sheep, goats, horses, pigs, dogs, cats, ferrets, camels, Llamas, deer, elk, elephants, rhinoceroses, foxes, coyotes, mink, primates, opossums, otters, seals, sea lions, hares, raccoons, bears, warthogs, large cats (lions, tigers, leopards, cheetahs and lynx) and several species of rodents (Miller, 2015; Musoke *et al*., 2015; Nugent, 2011; Palmer *et al*., 2012; Thoen *et al*., 2014).

Little is known about the susceptibility of birds to *M. bovis*, although they are generally thought to be resistant. Experimental infections have been reported in pigeon after oral or intra-tracheal inoculation and in crows after intraperitoneal inoculation (Fitzgerald *et al*., 2003). Some avian species, including mallard ducks, appear to be resistant to experimental infection (Clarke *et al*., 2006; Fitzgerald *et al*., 2003).

Cattle usually infect human beings, but other sources of infection have also been reported such as direct contact with a wound during hunting of infected game, and also from person to person when a person with pulmonary TB coughs or sneezes. Indeed, several cases of human tuberculosis caused by *M. bovis* have been described (Biet *et al*., 2005; CDC, 2005; Evans *et al*., 2007; Hlavsa *et al*., 2008) and there are also reports on human to human infection of *M. bovis* (Gallivan *et al*., 2015; Müller *et al*., 2013; Sunder *et al*. 2009). Human tuberculosis caused by *M. bovis* is clinically, radiologically and pathologically undistinguishable from the disease caused by *M. tuberculosis* (Cosivi *et al*., 1998; De la Rua-Domenech, 2006; Grange, 2001).

The disease caused by *M. bovis* is prevalent in most of Africa, parts of Asia, America and Middle Eastern countries. Nations classified as TB-free in domestic animals include Australia, Iceland, Denmark, Sweden, Norway, Finland, Austria, Switzerland, Luxembourg, Latvia, Slovakia, Lithuania, Estonia, the Czech Republic, Canada, Singapore, Jamaica, Barbados and Israel (OIE, 2014). Eradication programmes are in progress in Japan, New Zealand, United States of America, Mexico and some countries of central and South America (OIE, 2014). In South Africa, the existence of tuberculosis infection at the wildlife-livestock interface has complicated the control of bovine tuberculosis due to spill-over from wild animals to livestock (Michel *et al*., 2010) but spill-back from cattle to wild life is also a possibility.

1.2.2.3 Non-tuberculous mycobacteria

The non-tuberculous mycobacteria (NTM) or opportunistic mycobacteria are also referred to as atypical mycobacteria, mycobacteria other than tuberculosis (MOTT) or potentially pathogenic environmental mycobacteria (PPEM). They can survive in water and have been isolated in Europe, New Zealand and South Africa (Gcebe *et al*., 2013; van der Werf *et al*., 2014). NTM can complicate the interpretation of the tuberculin skin test in cattle as they can be responsible for non-specific sensitization of cattle to purified protein derivative (PPD) of bovine and avian tuberculin (Gcebe *et al*., 2013; Wagner and Young, 2004; Zeng *et al*., 2013). In humans, both *M. tuberculosis* and NTM can induce pulmonary infection with similar symptoms and pulmonary radiographic findings; these similarities have led to difficulty in distinguishing the infections clinically (Dailloux *et al*., 2006; Koh *et al*., 2002; McGrath *et al*., 2008). Adding to this, in primary health care with limited facilities using Ziehl-Neelsen as the main bacteriological identification of *M. tuberculosis*, NTM can easily be misdiagnosed as *M. tuberculosis*, therefore, relying on Ziehl-Neelsen staining alone can be misleading (Laifangbam *et al*., 2009; Padilla *et al*., 2005).

Mycobacteria are classified based on growth rate, pigmentation and clinical significance. Diseases caused by the non-tuberculous mycobacteria are named mycobacterioses (Han *et al*., 2007; Shitaye *et al*., 2009). They were considered as opportunistic pathogens often resistant to chemotherapeutic agents and not contagious (Pavlik *et al*., 2005; Slany *et al*., 2010; Tortoli, 2009). Over the past years, reports on isolation of the non-tuberculous mycobacteria from clinical samples from humans and animals have been increasing (Kabongo-Kayoka *et al*., 2015; Kankya *et al*., 2011; Malama *et al*., 2014; Muwonge *et al*., 2012; Tortoli, 2014).

NTM have been incriminated in the non-specific reactions observed on cattle reactors to tuberculin test during the interpretation process (De la Rua-Domenech *et al*., 2006; Kazda and Cook, 1988; Pavlik *et al*., 2005). In South Africa, they have been isolated from organs and lymph nodes of animals with lesions suggestive of tuberculosis (Gcebe *et al*., 2013; Kabongo-Kayoka *et al*., 2015). They have also been isolated from humans presenting clinical conditions suggestive of tuberculosis (Tortoli, 2014; Tortoli *et al*., 2009) in different countries such as the Czech Republic (Pavlik *et al*., 2005); China (Zeng *et al*., 2013), Zambia (Malama *et al*., 2014) and Uganda (Kankya *et al*., 2011). There are reports on the clinical importance of some NTM, namely *M. fortuitum* isolated from non-healing wounds in dogs and cats (Baral *et al*., 2006; Jang and Hirsh, 2002) and diseased fish (Zeng *et al*., 2013); *M. marinum* from snakes and bullfrogs (Maslow and Mikota, 2015). *Mycobacterium* *aurum* has been reported as the cause of infection in catheter-related blood-stream infections in humans (El Helou *et al*., 2013; Escalonilla *et al*., 1998; Koranyi and Ranalli, 2003). *Mycobacterium smegmatis* can also be found in normal human genital secretions (Best and Best, 2009). It was first isolated in 1884 by Lustgarden from syphilitic chancres but was found to be pathogenic for the first time by Vonmoos *et al*. (1986). *Mycobacterium smegmatis* has been identified as the cause of disseminated infection (Pierre-Audigier *et al*., 1997), pneumonia and infection of soft tissues (Best and Best, 2009; Ergan *et al*., 2004). In animals, *M. smegmatis* and other mycobacterial species were isolated from mastitis cases in cattle (Pardo *et al*., 2001; Thomson *et al*., 1988) and panniculitis pyogranuloma in cats (Ålander-Damsten *et al*., 2003). Different species of NTM have been isolated during experimental conditions and natural disease cases (Bercovier and Vincent, 2001). These NTM are sometimes referred to as non-pathogenic but it is preferable to refer to them as rapidly growing mycobacteria. The most important group widely spread in the environment and causing clinical diseases in various animal species apart from the obligate pathogens (*M. tuberculosis* complex) is the *M. avium* complex, also referred as *M. avium-intracellulare*. The *M. avium* complex includes *M. intracellulare*, *M. avium*, *M. paratuberculosis*, *M. lepraremurium* (pathogenic for rodents) and *M. avium* subsp*. silvaticum* subsp*. Nov,* known as wood pigeon bacillus.

Some members of the *M. avium* complex are saprophytes and others are pathogens of birds and mammals, including humans (Biet *et al*., 2005). They cause opportunistic infection in AIDS sufferers. There are different serovars involved in the disease process: serovars 1, 2 and 3 produce natural progressive disease in poultry and birds. In Europe, “Fowl tuberculosis” has been important and serovars 2 and 3 have been isolated from pig with lymphadenitis that were in close contact with fowls (Bono *et al*., 1995). This condition has also been reported in the USA. In South Africa, serovars 1, 4 and 8 are mainly responsible for pig lymphadenitis and serovars 4 and 8 occur frequently in the environment, in animal feed and bedding and on plants. In the Czech Republic, during a survey study conducted over 4 years by Pavlik *et al*. (2005), it was found that *M. avium* subsp. *avium* prevailed in cattle whereas *M. avium* subsp. *hominissuis* occurred in pigs.

Members of the *M. avium* complex produce visible growth in culture after two to three weeks. They grow at temperatures ranging from 20 to 45°C and form colonies that are pearly-grey, lemon-yellow or sometimes bright yellow and which emulsify easily (Biet *et al*., 2005). Methods to determine the relationship within the mycobacteria include immunological techniques, comparison of cell wall components, and comparison of homologous enzyme sequences, DNA/DNA homology, plasmid profiles, and restriction endonuclease analyses (BumJoon *et al*., 2004; Cousins *et al*., 2003; Roth *et al*., 2000; Stahl and Urbance, 1990). Comparative rRNA sequencing supports the classification of slow and rapid growers (Roth *et al*., 2000; Stahl and Urbance, 1990).

1.2.3 Pathogenesis of tuberculosis

Tuberculosis is not treated in animals and therefore, the focus of the pathogenesis will be on humans. *Mycobacterium tuberculosis* is a slow growing organism which is primary transmitted via the respiratory route but other routes can allow the entry of this bacterium, namely the oral route when using contaminated items or ingestion of contaminated food (gastro intestinal tract). Generally, the primary focus of infection is the lower zones of the lung (respiratory tract). The bacilli are taken up by lung macrophages but they can survive within the macrophages and grow to form the primary focus of infection and from there, enter the local lymphatic system and move within the body via the blood and lymphatic system. This phase of the disease is clinically silent and it is called “latent infection”; sometimes there is mild fever but in most cases, immunity develops within a few weeks and the patient becomes tuberculin positive (Leung *et al*., 2011).

Mycobacterium tuberculosis has a particular characteristic of being able to multiply intracellularly in the macrophages, evading many host defence mechanisms (Sakamoto, 2012). Acute inflammation is initiated by immune cells, macrophages which possess specialized receptors known as pattern recognition receptors (PRRs). Upon infection, macrophages are activated which enhances the ability of PRRs to recognize pathogen-associated molecular patterns (PAMPs) preserved on most pathogens (Walsh *et al*., 2013). When PRRs bind to PAMPs, the immune cells are activated to release inflammatory mediators (cytokines, plasma proteins and growth factors) to sustain the inflammatory process and recruit more cells to the site of injury or infection (Castellheim *et al*., 2009). Upon successful removal of the causative agents, the inflammatory response is quickly stopped and the repair mechanism is initiated by an active, coordinated process of resolution of the inflammatory episode (Walsh *et al*., 2013). In chronic inflammation, the inflammatory response is sustained, prolonged and further propagated causing damage to adjacent tissues and organs (Walsh *et al*., 2013).

Humans are as susceptible to *M. bovis* as to *M. tuberculosis*. Once a tuberculous lesion develops in an organ, the appearance and the course of the disease are the same irrespective of the causative agent (Cousins, 2001; Grange, 2001). Some mycobacterial infections remain localized, while others cause widely disseminated lesions. In general, depending on the route of infection, primary foci of tuberculosis occur in either the respiratory tract (95%) or gastro-intestinal tract. Other routes of infection for *M. bovis* in humans are percutaneous via damaged skin (for example, a meat inspector at an abattoir). Some factors do influence the length of time required for the disease to develop namely immune status, age, route of infection, strain of mycobacterium and the dose of mycobacteria (Shanmuganathan and Shanmuganathan, 2015).

The majority of individuals (90-95%) infected with *M. tuberculosis*, the main cause of TB, will never develop any clinical disease. There are risk predisposing factors that may lead to the development of clinical tuberculosis namely HIV infection, diabetes mellitus, gastrectomy, jejunoileal bypass surgery, dialysis dependent chronic kidney disease, renal failure, malnutrition, immunosuppression after solid organ transplantation, long-term corticosteroid treatment, tumour necrosis factor (TNF) inhibitors, advanced malignancy and to some extent genetic susceptibility among some individuals (Ferrara *et al*., 2012).

The most common form of tuberculosis is the chronic pulmonary form that is classified into three phases which include primary, post-primary dissemination or miliary and cell mediated immunity (Jacob *et al*., 2009; Sharma *et al*., 2005). To initiate infection, *M. tuberculosis* bacilli must be ingested by the macrophages. Bacilli that are not killed by the macrophages replicate inside them, killing the host macrophages with the help of CD8 lymphocytes; inflammatory cells are attracted to the area causing pneumonitis that coalesces into the characteristic tubercles observed histologically. The ability of the macrophages to kill mycobacteria is enhanced only after the development of delayed type hypersensitivity. The rapid development of the immune response following infection inhibits the growth of mycobacteria in lesions while they are still very small. Macrophages at the site of infection may be activated by direct contact with mycobacteria or as result of lymphokines.

The debris of necrotic macrophages sensitizes local lymphocytes. After sensitization, lymphocytes release lymphokines that attract, activate and increase the number of mononuclear cells at the site of infection. They also contribute to death of cells leading to caseous (cheese-like appearance) necrosis. Macrophages also play an integral part in the processing of mycobacterial antigens and the initiation of the immune response. Cell death caused by the delayed hypersensitivity reaction may be the result of lymphokines, toxic components of the bacteria, ischaemia, antigen-antibody reactions, hydrolytic enzymes and reactive oxygen intermediates released from necrotic macrophages and neutrophils, and tumour necrosis factor (Ahmad, 2010; Lee *et al*., 2011; Leung *et al*., 2011; Parandhaman and Narayanan, 2014; Young, 1993).

In the early weeks of infection, some infected macrophages can migrate to regional lymph nodes where they access the bloodstream. Organisms may then spread to any part of the body. In the pulmonary form, the lesions occur primarily in the lymphoid tissues whereas both the lung parenchyma and the lymph nodes are affected in the respiratory system. Although hematogenous spread of tubercle bacilli from the lung is seldom, hematogenous dissemination accounts for the occurrence of extrapulmonary involvement of lymph nodes, kidneys, reproductive organs, bones and the gastrointestinal tract. Local features vary according to the organ involved.

Hematogenous dissemination is less likely in patients with partial immunity due to vaccination or to prior natural infection with *M. tuberculosis* or environmental mycobacteria (non-tuberculous). In 95% of cases, after three weeks of uninhibited growth, the immune system suppresses bacillary replication, usually before symptoms develop (Leung *et al*., 2011; Young, 1993). Foci of bacilli in the lungs or other sites resolve into epithelioid cell granulomas, which may have caseous and necrotic centres. Tubercle bacilli can survive in this state for years; the balance between the host's resistance and microbial virulence determines whether the infection ultimately resolves without treatment, remains dormant, or becomes active (Delogu *et al*., 2013; Leung *et al*., 2011; Sakamoto, 2012; Young, 1993). Infectious foci may leave fibro-nodular scars in the apices of one or both lungs (Simon foci, which usually result from hematogenous seeding from another site of infection) or small areas of consolidation (Ghon foci).

The initial possible infection is the contact between the inhaled bacteria and lung alveolar epithelial mucosal cells followed by reaching macrophages. When inhaled, droplet nuclei that are less than 100 nm usually travel through the airway until they reach the alveoli (Inderlied, 2004; Leung *et al*., 2011). Once the bacteria enter the macrophage, they locate in the mycobacterial phagosome (Inderlied, 2004; Sakamoto, 2012). Unlike normal phagocytosis, during which the phagosomal content is degraded upon fusion with lysosomes, the mycobacteria block this process (Inderlied, 2004; Sakamoto, 2012). The inhaled bacilli are phagocytosed, processed and presented by alveolar macrophages to the lymphocytes (Sakamoto, 2012). The probability of infection depends on the ability of the mycobacterium to survive within the macrophages, in this way evading many host defence mechanism (Figure 1.1). At this stage unless the patient receives prophylaxis, symptomatic disease eventually occurs in 5-10% of infected patients.

The maturation of mycobacterial phagosomes is stopped and therefore, these cells are unable to eliminate bacilli by fusion of phagosomes with lysosomes. The inhibition of phagosome maturation by mycobacteria may be reverted by cytokines, such as interferon-gamma (IFN-γ) and tumour necrosis alpha (TNF-α) which also stimulate microbicidal mechanisms, including the production of reactive oxygen and nitrogen intermediates (Chan *et al*., 1992). Nitrogen intermediates play a protective role to some extent (Nicholson *et al*., 1996); reactive oxygen intermediates namely hydrogen peroxide produced by the macrophages activated by cytokines, have a mycobactericidal activity (Walker and Lowrie, 1981). In addition, the tubercles of bacilli present other molecules such as lipoarabinomannan (LAM), phenolic glycolipid I and thiols such as mycothiol (MSH) which work as oxygen radical scavenger molecules (Chan *et al*., 1991).

A Ghon focus with lymph node involvement is a Ghon complex, which, if calcified, is called a Ranke complex. The tuberculin skin test and interferon-gamma release blood assays (IGRA) become positive during the latent stage of infection. Sites of latent infection are dynamic processes. Extra-pulmonary TB at any site can sometimes manifest without evidence of lung involvement. Lymphadenopathy TB is the most common extra pulmonary presentation; however, meningitis is the most feared because of its high mortality in the very young and very old (Donald *et al*., 2005; Marx and Chan, 2011).

1.2.4 Clinical signs of tuberculosis

Clinical signs observed may include a persistent cough, night sweats, lassitude, weight loss, malaise, fever and anorexia, dyspneoa, chest pain, signs of chest disease (Churchyard *et al*., 2014; Inderlied, 2004).

At every stage during the course of the tuberculous process, the character of the lesions depends on the fluctuation between cell mediated immunity and the delayed type hypersensitivity. Adding to this, the development of either an exudative or a proliferative response depends on the concentration of bacterial antigen, with low concentrations causing proliferative lesions, whereas high concentrations result in an exudative response (Delogu *et al*., 2013; Inderlied, 2004).

1.2.5 Diagnostic tests

There are different traditional methods for the diagnosis of tuberculosis which include the tuberculin skin test, culture and smear microscopy. Each of these methods has limitations. In humans, one of the challenges of the tuberculin test is that prior vaccination with BCG may result in a false positive result even many years later. The culture method, although sensitive, is time-consuming, and takes 6-8 weeks to obtain results (Cosivi *et al*., 1998; Cousins, 2001). In laboratories that only process human samples, *M. bovis* is often missed as the routine growth medium used (LJ with glycerol) does not contain pyruvate which is a substrate that enhances the growth of *M. bovis* (de Kantor *et al*., 2008; Grange, 2001). Furthermore, the suspected cases might be missed due to delay. Staining of infective organisms by the Ziehl-Neelsen (ZN) technique in sputum has both the problems of sensitivity and specificity as there are many other organisms that are also acid-fast and therefore one cannot rely on ZN staining as the only method of identification. Although, time-consuming, the culture method and ZN staining are still used for the confirmation of acid-fast organisms and diagnosis of tuberculosis (Bhalla *et al*., 2015; Kabongo-Kayoka *et al*., 2015; Ryu, 2015).

Other time-consuming methods include phenotypic tests based on biochemical substrates, mycolic acid analysis and narrow spectrum nucleic acid probes. Bacteria are identified based on biochemical and physiological features. These methods are sometimes not able to differentiate between different *Mycobacterium* species. Puttinaowarat *et al*. (2002) reported on combining PCR followed by reverse cross blot hybridisation (RCBH) which was highly specific for *Mycobacterium* spp. and identified bacteria to the species level with a detection limit of 100 fg which is equivalent to 20 mycobacterial cfu. For an improved differentiation of *Mycobacterium tuberculosis* strains, Yokoyama *et al*. (2007) proposed the use of a combination of variable number of tandem repeat loci.

Chemotaxonomic methods include analysis of the quinone system, fatty acid profiles, polar lipid patterns, polyamine patterns, whole cell sugars, peptidoglycan diaminoacids, analytical fingerprints methods and cellular protein patterning (Butler and Guthertz, 2001; Layre *et al*., 2011). Other approaches include PCR and sequencing technologies (Cloud *et al*., 2002). The microSeq 500 system is a commercial kit based on the sequencing and analysis of the gene 16S rRNA; it has limitations as it requires the use of additional databases such as the Ribosomal Differentiation of Medical Microorganisms (RIDOM) to precisely identify subtypes of strains not currently in the MicroSeq library (Fontana *et al*., 2005). Gene sequencing allows the analysis of phylogenetic relationships of bacteria, nucleic acids hybridization, G+C content determination, restriction fragment length polymorphism (RFLP), macrorestriction analysis and random amplified polymorphic DNA (RAPD) (Jansen *et al*., 2002; Laurent *et al*., 1999).

Advances in the molecular field and understanding of the genetic structure of mycobacteria has led to the development of techniques and tools that can be used to confirm the identity of isolates from clinical samples and study of the epidemiology of tuberculosis. PCR-based methods are available as diagnostic and confirmatory tests for tuberculosis and are expected to detect as few as 1-10 organisms, but its use in surveillance and epidemiological studies might not be possible (Ryu, 2015).

Molecular typing methods such as IS6110-RFLP and spoligotyping can be used for identification of outbreaks and can facilitate contact tracing of tuberculosis. IS6110-RFLP is considered a gold standard for the molecular typing of *M. tuberculosis* but this has provided limited discrimination among *M. bovis* populations where the majority of the isolates harbour only one or a few IS copies (Haddad *et al*., 2004). IS6110-RFLP has a high discriminative power and reproducibility but the technique requires a large amount of DNA and is therefore restricted to the mycobacterial cultures which take around 20-40 days to obtain sufficient DNA needed. Moreover, this technique is also demanding technically, is expensive and requires sophisticated software for result analysis. For *M. bovis*, PCR-based spoligotyping (Kamerbeek *et al*., 1997) has been widely used to genotype isolates; it is highly reproducible and represents the first universally recognized typing system for *M. bovis* but other studies have showed a limited discrimination power of this method (Hilty *et al*., 2005; Roring *et al*., 2002; Skuce *et al*., 2002).

Spoligotyping (spacer oligonucleotide typing) is a method that can be simultaneously used for detection as well as typing of the *M. tuberculosis* complex. It is a PCR-based method, which relies on the amplification of the spacers using the primers DRa and DRb, which allow the amplification of the highly polymorphic Direct Repeat (DR) locus, the whole DR genome (Haddad *et al*., 2001). The DR region in *M. bovis* BCG contains direct repeat sequences of 36 bp, which is interspersed by the non-repetitive DNA spacers of 35-41 bp in length. In other *M. tuberculosis* complex strains, the number of DR elements varies significantly and the majority of the *M. tuberculosis* strains contain one or more IS6110 elements in the DR region. A main feature for *M. bovis* is the lack of spacers 39 to 43 (Aranaz *et al*., 1996).

Genetic typing of *M. bovis* has contributed to a greatly improved knowledge of inter-bovine and inter-species transmission of bovine tuberculosis (Durr *et al*., 2000). The understanding of genetic typing is important for the effective management of bovine tuberculosis control schemes and the wildlife–livestock interface in countries where wildlife reservoirs for *M. bovis* have been identified, including South Africa (Haddad *et al*., 2004; Vos *et al*., 2001). The most widely used DNA typing techniques for *M. bovis* include IS6110 and PGRS restriction fragment length polymorphism (RFLP) typing, spoligotyping and variable number of tandem repeat (VNTR) typing (Sola *et* *al*., 2003; Wirth *et al*., 2008). In South Africa, spoligotyping and VNTR typing have been used (Hlokwe *et al*., 2014; Michel *et al*., 2008).

Other methods for rapid molecular epidemiology investigation include six VNTR loci, A through F (ETR-A,-B, -C, -D, -E and –F), which were reported to be more discriminative than spoligotyping (Frothingham and Meeker-O'Connell, 1998). A method with more genetic markers, MIRU-VNTR was described and comprised ETRS, mycobacterial interspersed repetitive units (MIRUs) and VNTRS (Roring *et al*., 2002; Skuce *et al*., 2002). Combining methods can reduce their respective limitations and improve the identification of mycobacteria. A combination of spoligotyping and MIRU-VNTR provides a discrimination power greater than 90% (Roring *et al*., 2002).

The similarity of mycobacteria can be based on the 16S rDNA most conserved region but further differentiation is obtained with the concatenation of all four genes namely ITS, 16S rDNA, rpoB and hsp65 gene sequences that require high identification resolution (Cloud *et al*., 2002).

The whole cell MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time Of Flight) aided with diagnostic proteins is robust for *Mycobacterium* spp. phenotypic characterization (Buckwalter *et al*., 2016; Wang *et al*., 2012) whereas for genotypic characterization, the multiple-locus sequence analysis of all four genes are required for the complete identification at the species level of any *Mycobacterium* spp. (Cloud *et al*., 2002; Kabongo-Kayoka *et al*., 2015). Mycobacteria can also be differentiated or identified based on the composition of the longer chain alpha mycolic acids of their cell wall (C80). Each mycobacterial species possesses a characteristic profile of alpha-mycolic composition and that the use of normal phase liquid chromatography with thermally assisted hydrolysis and methylation followed by gas chromatography-mass spectrometry could give rapid and very precise information for the identification of pathogenic and non-pathogenic mycobacterial species (Mourão *et al*., 2016).

Phylogenetic analysis is conducted to find similarities between organisms (analysis of changes that have occurred in different organisms during evolution), to understand or find relationships between an ancestral sequence and its descendant and estimate time of divergence between a group of organisms from a common ancestor. This evolution is illustrated as a phylogenetic tree. There are different methods to build phylogenetic trees, namely neighbour joining, the parsimony method and maximum likelihood (Tamura *et al*., 2011).

1.2.6 Challenges in the treatment of tuberculosis

Tuberculosis treatment is based on the use of multi-drug treatment where usually three to four anti-TB drugs are combined. The cocktail of rifampin (RIF), isoniazid (INH), pyrazinamide (PA) and ethambutol (EMB) are the recommended first line drugs. Second line drugs, in case of organism resistance or heavy side effects, include fluoroquinolones, aminoglycosides or streptomycin (CDC and Society, 2003; Warner and Mizrahi, 2006). The emergence of TB strains resistant to more than one drug is a concern. Factors that may lead to the development of resistance include patients failing to adhere to the regular administration of the correct dosage due to the long duration and toxic effects of the drugs; other factors such as immunosuppressed patients may contribute to the development of resistant strains. The molecular basis of MDR TB has been well documented (Bosne-David *et al*., 2000; Cingolani *et al*., 1999; Johnson *et al*., 2007; Musser, 1995; Sreevatsan *et al*., 1997). Mutations that occur in the target genes of *M. tuberculosis* and *M. bovis* strains are the most common mechanisms of resistance encountered for primary antimycobacterial agents such as RIF and INH.

MDR strains of TB do not respond to treatment with rifampin and isoniazid which are the first line drugs in the initial treatment regime of TB whereas XDR-TB strains are not only resistant to both first line drugs, rifampin and isoniazid, but also to one of the three second line drugs, for example fluoroquinolones. The normal mechanism of activity is either hampered or inhibited (Figure 1.7). MDR-TB and XDR-TB infections are difficult to treat and require a long duration of treatment. As the disease is chronic and develops slowly, lesions might already be established when the disease is clinically diagnosed and surgical removal of necrotic tissues is sometimes required as permeability of drugs into dead tissues is sometimes not possible (Kaplan *et al*., 2003). The cost to treat such cases is high and requires long courses of antibiotic treatment with possible adverse consequences due to toxic side effects of more expensive drugs. According to the World Health Organization (2015), it is estimated worldwide that 3.3% of new TB cases and 20% of previously treated cases are MDR-TB. Eastern European and central Asian countries continue to have the highest levels of MDR-TB. South Africa was among the six countries with the highest incident cases in 2014 (WHO, 2015) and it is still on the list of the countries with a high TB burden. Other countries are India, Indonesia, China, Nigeria and Pakistan. There is a need for alternative treatment with fewer toxic effects, possibly using natural resources.

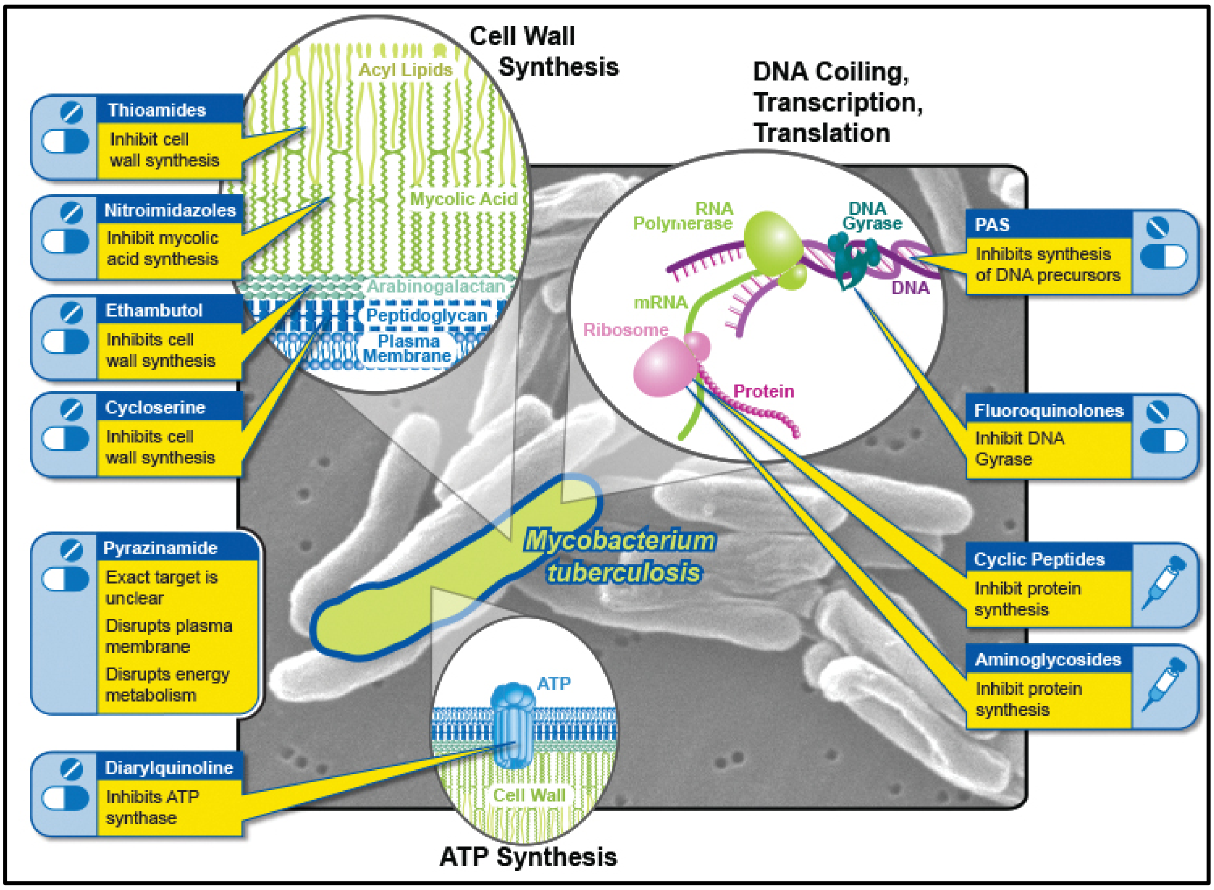


Figure 1.7: Mechanism of activity of antiTB drugs

Source: courtesy of www.slideshare.net

1.2.7 The use of traditional medicine as an alternative or complementary medicine

Alternative medicine is a term referring to a broad spectrum of healthcare practices, namely acupuncture, chiropractic, herbalism, homeopathy and naturopathy, whereas complementary medicine refers to the use of treatments drawn from alternative medicine to complement allopathic medicine (Cleary-Guida *et al*., 2001; Ernst and

White, 2000). This is an unfortunate clumping of practices because herbalism is based on scientifically understood pharmacological activities of compounds present in plants (Craker and Gardner, 2003; Cushman and Hoffman, 2004; Hoffmann, 2003).

Despite the advances made in the treatment of many microbial infections caused by microorganisms and other diseases with conventional drugs, there has been a global cultural revival towards more natural methods of healing. The use of traditional plant-based medicine remains ingrained in the healing practices of many developing countries (Makunga *et al*., 2008). It has been reported that about 80% of communities of African origin rely on herbal medicine for primary health care purposes (Dubey *et al*., 2004; Mahomoodally *et al*., 2010). The huge reliance of African people on traditional medicine can be attributed to availability, accessibility, affordability, extensive traditional knowledge and expertise within local people (Gurib-Fakim, 2006). Conventional medicine has been accepting the use of traditional medicine once it has been scientifically validated (Gilani, 2005).

Prior to modern medicine, people have been using medicinal plants for the treatment of various ailments for centuries (Briskin, 2000). Plant-derived medicines are used for primary health care to treat diseases such as cancer, diabetes, infectious diseases and many others (Zahin *et al*., 2010). In South Africa, both Western and traditional systems of medicines co-exist. Three hundred years ago, the Western system was introduced by European settlers and traditional medicine was established from Palaeolithic times (Van Wyk *et al*., 1997). It is estimated that 27 million South Africans rely on traditional medicine for their primary health care needs (Mander *et al*., 2007; Van Vuuren, 2008).

1.2.8 New lead compounds for drug discovery and development

There has been an increased interest in investigating medicinal plants for the discovery of new lead compounds for potential drug development (Eloff and McGaw, 2014; Rout *et al*., 2009). Indeed, extracts derived from medicinal plants have been used for a very long time for the management and prevention of many diseases. Despite the rise of synthetic chemistry as an integral part of lead discovery process, natural products still play a major role as starting material for drug discovery (Katiyar *et al*., 2012; Saleem *et al*., 2010). According to Newman and Cragg (2007), of the

974 small molecules used as therapeutic drugs, 63% were natural product-derived or semi-synthetic derivatives of natural products. The number continues to increase annually, such that 48.6% of anti-cancer drugs are either natural products or plant-derived entities (Newman and Cragg, 2010). Veeresham (2012) stated that 11% of the 252 drugs presented by WHO originates from flowering plants.

Emergence of drug-resistant organisms, ease of accessibility of traditional medicines and high cost of conventional health care systems as well as traditional beliefs are among the factors that continue to promote and sustain interest in alternative forms of medical care in non-industrialized countries (Cerella *et al*., 2013; Gurib-Fakim, 2006) including developed countries (WHO, 2002). It is further stated that 75% of people living with HIV/AIDS use traditional medicine for therapeutic purposes in South Africa (WHO, 2002). Plant-derived substances are used as therapeutics in many communities in developed countries for primary health care needs. As much as 50% of the populations of Europe and North America use plant-derived substances as supplements or primary therapeutics (WHO, 2002). Though the pace of discovery of potential lead compounds and their development remains exceedingly slow, plants are still good sources because of their ability to synthesize secondary metabolites in response to microbial infection (Cowan, 1999). Although the efficacy of plant-derived extracts for disease treatment has been known for centuries, the first isolation of a bioactive compound from a medicinal plant species was only achieved in the 19^{th} century (Phillipson, 2001). Despite the implied potential of natural products, only a fraction of medicinal plant species has been tested for bioactivity (Gautam *et al*., 2007). The rich diversity of compounds with novel structures and biological activity make them a source of different types of drugs.

Plant extracts contain multiple component mixtures of active, partially active and inactive substances. This includes primary metabolites, such as sugars and amino acids, found in all plants and secondary metabolites, while some are found only in a particular genus or species (Hussain e*t al*., 2012; Vanisree *et al*., 2004). This explains the variation in isolating a compound from a specific species within a genus or family while it may be absent in the rest.

The secondary metabolites and pigments that have potential therapeutic actions in humans can be refined to produce drugs (Pandey *et al*., 2011). The activity of the plant extract is the sum of the activities of the individual phytochemicals as well as the interaction between them. This approach has led not only to the introduction of many important drugs but still forms the basis of studying lead compounds from plants (Efferth and Koch, 2011; Habib-ur-Rehman *et al*., 2007). Some of the drugs that are currently used originated from the diverse structures and the intricate carbon skeletons of natural products (Rout *et al*., 2009). Innovations in phytochemical analysis and the ability to synthesize them have allowed scientists to modify structures in order to enhance certain characteristics such as solubility, efficiency and stability in the human body (Ji *et al*., 2009). Out of many families of secondary metabolites, the alkaloids have contributed the largest number of drugs to the modern pharmacopoeia. The terpenoids (including steroids) have made equally important contributions to human health (Raskin *et al*., 2002; Wang *et al*., 2005). Tannins, polyphenols, saponins, flavonoids and phytates have been isolated and studied at the cellular and molecular levels but in general, these compounds were not useful in treating antimicrobial infections (Singh *et al*., 2003).

Interesting compounds have been isolated from plant species, bacteria and fungi and have a broad spectrum of biological actions. Among many others, naphthoquinones have been reported for their antibiotic, antiviral, anti-inflammatory, antipyretic and anti-proliferative properties (Babula *et al*., 2009).

Plant species still serve as a rich source of many novel biologically active compounds, as very few plant species have been thoroughly investigated for their medicinal properties (Gautam *et al*., 2007; Jiménez-Arellanes *et al*., 2013; Jimenez-Arellanes *et al*., 2003; Newman *et al*., 2003). Several plants such as *Buddleja saligna* have been screened for their antimycobacterial activity (Bamuamba *et al*., 2008). Several plant extracts have been found to be active against *M. tuberculosis* and other model mycobacteria such as *M. aurum*, *M. smegmatis* and *M. fortuitum* (Aro *et al*., 2015; Gautam *et al*., 2007; Green *et al*., 2010; Kabongo-Kayoka *et al*., 2016).

Ethnobotanical information has also shown that plants claimed to treat diseases such as TB inhibit the growth of *Mycobacterium tuberculosis* in culture (Tabuti *et al*., 2010). *Mycobacterium aurum* has been reported as a good model for *M. tuberculosis* due to its low pathogenicity potential and rapid growth rate. *Mycobacterium aurum* has the same sensitivity profile as *M. tuberculosis* as their fatty acid chains in the cell wall are of similar length which plays an important role in susceptibility of mycobacteria (Chung *et al*., 1995). *Mycobacterium aurum,* therefore, has been used frequently in drug screening where the actual pathogens cannot be grown without risk of contamination and infection (Chung *et al*., 1995; Mukanganyama *et al*., 2015; Shawar *et al*., 1997). Diospyrin (from *Diospyros montana* Roxb) has been explored as a potential drug efflux inhibitor for the treatment of tuberculosis (Mukanganyama *et al*., 2015). Diospyrin and some of its derivatives were found to inhibit mycobacterial growth and enhanced accumulation of drugs inside the bacteria (Mukanganyama *et al*., 2015). Other secondary metabolites such as alkaloids have been reported to have antimycobacterial activity (Camacho-Corona *et al*., 2009).

1.2.9 Antimycobacterial activity of South African medicinal plants and bioactive compounds

In South Africa, many people still rely on traditional medicine for their primary health care and due to socio-economic challenges some of those medicines are sold in informal markets (McKean and Mander, 2007). Therefore, the scientific validation of the biological activities of South African plants is still justified though a considerable amount of work has been reported on the antimicrobial activity to verify some of the traditional claims (Fennell *et al*., 2004; Light *et al*., 2005). Very few have reported antimicrobial activity against *Mycobacterium* spp. causing tuberculosis.

The Phytomedicine Programme at the Faculty of Veterinary Science at the University of Pretoria is a multidisciplinary and collaborative research programme that investigates the use of medicinal plants growing in Africa for the benefit of its people and animals. Acetone leaf extracts of more than 500 southern African plant species have been evaluated for activity against eight important bacterial and fungal pathogens for general antimicrobial activity; investigating the possibility that extracts could be used in the primary health care of rural people (Pauw and Eloff, 2014). Some leaf extracts of different plant families were also evaluated specifically against *Mycobacterium* spp. for antimycobacterial activity aiming at finding potential leads for the treatment of tuberculosis. These acetone leaf extracts included *Euclea* species and plants from the Rubiaceae and Anacardiaceae families (Aro *et al*., 2015; Kabongo-Kayoka *et al*., 2016; McGaw *et al*., 2008a). Other studies have also reported potential antimycobacterial activity for plants belonging to families such as *Scutia buxifolia* (Rhamnaceae); *Terminalia sericea* (Combretaceae); *Warburgia salutaris* (Canellaceae); *Asclepias fruticosa* (Apocynaceae) and others (Boligon *et al*., 2012; Green *et al*., 2010; Mariita *et al*., 2010).

Essential oils, alkaloids, terpenoids, phenolic acids, peptides, flavonoids, tannins and coumarins represent the principal groups of compounds extracted from plants with antimycobacterial properties reported in the literature (Andrade-Ochoa *et al*., 2015; Kishore *et al*., 2009; McGaw *et al*., 2008a; Santhosh and Suriyanarayanan, 2014). Many bioactive compounds have been isolated from plant species which had promising activities during the preliminary phase, from different plant families (Eloff *et al*., 2008). The bioactive compounds isolated to date were relatively non-polar. Therefore, the tendency is to focus on non-polar compounds due to complexity of phytochemical compounds in a plant. Although isolation of most compounds has been reported elsewhere, some of the compounds have been isolated from leaves tested for antimicrobial activity and cytotoxicity for the first time in the Phytomedicine Programme. For example, two novel terpenoid compounds have been isolated from *Combretum imberbe* and *Combretum padoides* (Katerere *et al*., 2003).

1.2.10 Selection of potential medicinal plants for drug development

There are several strategies for selecting medicinal plants suitable for pharmacological studies. These include traditional use (ethnobotany or ethnopharmacology), chemical content, toxicity, randomized selection or a combination of several criteria (Blondeau *et al*., 2010; Rates, 2001; Santhosh and Suriyanarayanan, 2014; Schwikkard and Mulholland, 2014; Soejarto *et al*., 2012; Ramesha *et al*., 2011; Williamson *et al*., 1996). The ethno pharmacological approach is often the preferred method because of the rich information on how medicinal plants are used by different ethnic groups. The preparation procedure by the traditional practitioners provides vital information or indication of the best extraction method and the mode of administration when the chosen plant species has promising *in vitro* pharmacological activity that warrants further investigation using animal models. The best way of selecting potential medicinal plants is to use a combination of several approaches (Rates, 2001).

Another imperative demand imposed on scientific investigation is that the result should be repeatable (Hedberg, 1993). Medicinal plants harvested from the wild generally vary in quality and consistency of the active secondary metabolites due to genetic, ecological and environmental differences (Bopana and Saxena, 2007). Factors such as plant age, seasonal variation and geographical deviation contribute towards variation in pharmacological activity. Medicinal plants are in great demand in developing countries and at times mature trees or plants cannot be found, then the young ones are used, which results in inconsistency of plant material of the same species and conditions (Street *et al*., 2008). In order to develop an antimicrobial drug, it is important to be able to achieve the same level of activity in the source material. For sustainability, plant leaves are a more viable option compared to the use of other parts of the plants such as root, bark, stem or fruits, especially if the bioactive phytochemicals are the same. The extinction of vulnerable species is also a danger when using vital parts such as roots, bark and stem. This explains why botanical gardens have become sources of collection of plant material used in many scientific investigations.

Despite many publications on antimicrobial activity of plants extract, it has not yet led to the development of a new commercial antimicrobial compound (Gertsch, 2009). One of the reasons for this failure could be that scientists selected plant species for research based on traditional knowledge. Traditional healers have mainly water available as an extractant while it has been established that aqueous extracts generally have little to no antimicrobial activity (Eloff *et al*., 2005; Kotzé and Eloff, 2002). Therefore, random screening of antimicrobial activities of acetone leaf extracts of more than 500 representative tree species belonging to different families was undertaken in the Phytomedicine Programme against eight microbial pathogens including *Mycobacterium smegmatis* as leads for in-depth research (Pauw and Eloff, 2014). Based on the good antimicrobial activity of plant species from some families including the Anacardiaceae, the selection was extended to other members of the same family to evaluate them for antimycobacterial activity (Kabongo-Kayoka *et al*., 2016).

1.3 PROBLEM STATEMENT

Tuberculosis has increased worldwide during the last century and the incidence of this contagious and deadly disease reached a peak in 2005 while the total number of new TB cases is still increasing. The positive case numbers of human immunodeficiency virus (HIV) and multidrug resistant patients also increased (Dorman and Chaisson, 2007; WHO, 2007; Shah *et al*., 2007). Besides TB, other species of non-tuberculous mycobacteria causing disease have also been isolated from non-HIV and HIV-affected patients including *M. avium* (Biet *et al*., 2005; Katale *et al*., 2014).

In humans, childhood acquired immunity for TB has been obtained through vaccination using the Bacille Calmette Guerin (BCG) vaccine which is an attenuated bovine strain. But, studies have reported that the vaccine is only partially effective (Andersen and Doherty, 2005; WHO, 2015; Trunz *et al*., 2006; Young and Dye, 2006). To explore possibilities of drug development and vaccines, it is essential to make accurate diagnosis and conduct experiments using not only reference strain cultures from known culture collection databases such as the American Type Collection Culture (ATCC) or National Collection Type Cultures (NCTC) but also using current isolates as mutations might take place in this ever changing environment. The continuous monitoring of *Mycobacterium* spp. infections in humans as well as in animals for any change in antimicrobial patterns is therefore necessary to evaluate the development of resistance against existing antimicrobial drugs, bearing in mind that tuberculosis is a zoonotic disease. In 2010, the WHO stated that managing and responding to risks related to zoonoses and some high impact diseases require multi-sectoral and multi-institutional cooperation. Strengthening collaboration in human and animal health is essential. The Food and Agriculture Organization (FAO), Office International des Epizooties (OIE) and WHO (2010) suggested among others the development of a joint framework to address gaps and strengthen collaboration in human and animal health laboratory activities. Therefore, the approach in this research project has been multi-inter-transdisciplinary (MIT).

For more than 50 years, there has not been a new molecule discovery of antibiotics. Most new drugs are modifications of the existing antibiotics except the newly reported drug bedaquiline (Biuković *et al.*, 2013; Diacon *et al.*, 2012, 2009; Sacksteder *et al.*, 2012). The excessive use of antibiotics in treatment, the food industry and improper administration has led to an increase of antibiotic resistance in the market (Burnet *et al.*, 2000). Treatment of tuberculosis has become a challenge due to emerging multi-drug resistant strains (Tomioka, 2006).

The long duration (6-9 months) of treatment and the adverse effects of chemotherapy are some of the reasons for lack of adherence to the completion of the treatment regime. This situation has led to the increased emergence of MDR-TB and XDR-TB. In South Africa, MDR-TB has also been identified (Wilkinson *et al.*, 1996) and tuberculosis was declared an emergency after an outbreak due to an extremely deadly resistant strain (XDR-TB) that was responsible for morbidity and mortality in the KwaZulu-Natal Province (Andrews *et al.*, 2008; Basu *et al.*, 2007). It is therefore imperative to conduct periodic studies on characterization of mycobacterial species, antibiogram profiles and identification of other potential antibiotic drugs from natural resources.

During the first global XDR-TB task force that WHO held in Geneva from 8-9 October 2006, the following areas were identified as urgent research priorities: epidemiological characterization, relationship between strains and resistance, virulence and drug resistance, full understanding of the current disaster and *Mycobacterium tuberculosis* identification and drug susceptibility testing as well as needs to identify alternative treatments besides current available drugs (WHO, 2007). Plants have formed the basis of many traditional medicine systems throughout the world for centuries and continue to provide mankind with new remedies. Numerous useful drugs were developed from lead compounds discovered from medicinal plants. This strategy remains an essential route to new pharmaceuticals (Newman *et al.*, 2003). There are few publications showing activity of certain plants against the pathogenic mycobacteria, non-pathogenic *M. bovis* BCG and rapidly growing or non-tuberculous Mycobacteria namely *M. aurum*, *M. smegmatis and M. fortuitum*. Further investigation with emphasis on pathogenic mycobacteria, namely *M. bovis* and *M. tuberculosis,* is still needed to provide a broader database of potential plant species as candidates for possible antimycobacterial drug development.

1.4 AIMS AND OBJECTIVES

This study comprised two phases: phase I included the isolation of *Mycobacterium* spp. from samples of animals suspected of being infected with TB after tuberculination or showing lesions suggestive of tuberculosis and characterization of some of the isolates. During phase II, selected plant species of the family Anacardiaceae were tested for antimycobacterial activity using some isolates from phase I and strains of non-tuberculous mycobacteria.

The aims of this research project were:

- Phase 1: To isolate and characterize *M. bovis* and *M. avium* strains from animal samples;
- Phase 2: To test some first-line antimicrobial drugs and selected plant extracts against isolates from phase I and strains of non-tuberculous mycobacteria for antimicrobial sensitivity.

The abovementioned aims were achieved through the following objectives:

- To process samples from animals suspected of tuberculosis and isolate *Mycobacterium* species;
- To identify and characterize the above isolates;
- To test some of these isolates for antimycobacterial sensitivity using first-line drugs;
- To select 15 plant species from a list of previously screened plants (in the family Anacardiaceae) with good activity against *Mycobacterium smegmatis*.
- To collect the leaves from the selected plants and prepare crude acetone extracts from dried leaf materials;
- To determine in vitro antimycobacterial activities and cytotoxicity of crude acetone extracts of the selected plant species;
- To select the most active extract for bioassay-guided isolation of antimycobacterial active compounds;
- To perform solvent-solvent fractionation of the most active crude acetone extract and run a second screening on the fractions for antimycobacterial activity;
- To select the most active fraction/s for compound isolation;

- To isolate and determine the molecular structure of at least one compound responsible for antimycobacterial activity;
- To determine the antimycobacterial activity and cytotoxicity of the isolated compounds.

1.5 HYPOTHESIS

New *Mycobacterium* species can still be isolated in the current ever-changing environment. The selected plant extracts may contain chemical compounds with *in vitro* antimycobacterial activity.

1.6 SCOPE OF THE THESIS

1.6.1 Isolation and characterization of mycobacteria

To explore possibilities of drug development and vaccines, it is essential to make accurate diagnosis and conduct experiments using not only reference strain cultures but also using the current clinical isolates as mycobacterial mutations might have occurred in this ever-changing environment. The treatment of tuberculosis is still a challenge due to emergence of MDR and XDR strains. Therefore, there is a need to characterize and establish the genetic diversity of the current isolates used in bioassays while exploring the development of alternative drugs for tuberculosis. The continuous monitoring of *Mycobacterium* spp. infections in humans as well as in animals for any change in antimicrobial patterns is therefore necessary to evaluate the development of resistance against existing antimicrobial drugs, bearing in mind that tuberculosis is a zoonotic disease. Samples from slaughtered animals suspected of being infected with tuberculosis or presenting lesions suggestive of tuberculosis were processed for *Mycobacterium* spp. isolation and characterization.

1.6.2 Antimycobacterial activity of plant extracts

Previous screening of some tree species from the Anacardiaceae family revealed the presence of good antimicrobial activity against *Mycobacterium smegmatis* (Pauw, 2014). Leaf extracts of 15 Anacardiaceae tree species were screened for antimycobacterial activity against the pathogenic *Mycobacterium bovis*, multidrug resistant *M. tuberculosis,* drug sensitive *M. tuberculosis* (H37Ra), *M. bovis* BCG vaccine strain and strains of non-tuberculous mycobacteria, namely *M. aurum, M. fortuitum* and *M. smegmatis.*

1.6.3 Cytotoxicity assay of plant extracts

For a plant extract or compound to be considered for drug development or as an alternative for therapeutic use it should be more toxic to the target organism than the patient’s cells. The cytotoxicity of the active crude extract, its fractions and pure active compounds was investigated and the selectivity index was calculated to achieve this aim.

Once the antimycobacterial activity of the 15 plant extracts was established, isolation of the pure active compounds from one of the most active and least toxic plant species on normal cells was carried out. Cytotoxicity was evaluated using Vero monkey kidney cells, human hepatoma liver cells and murine macrophages.

1.6.4 Isolation, purification and identification of the active compound(s) from *Searsia undulata*

Out of the 15 plant species tested for antimycobacterial activity, 5 had moderate to good activity. Of these five plant species; *Searsia undulata* had good antimycobacterial activity and low cytotoxicity to normal cells (but was toxic to the C3A hepatoma cells). According to the literature review, leaves of *Searsia undulata* are chewed by the Nama (Khoisan) for chest colds and leaf decoctions are administered for *post partem* problems. The plant is a shrub widely distributed along the west coast of the Cape and occurs in a range of different habitats (Van Wyk *et al*., 1997). This information supported the decision to focus on this plant species first.

The objective was to isolate active compound(s) and evaluate the minimum inhibitory concentration with the different fractions and finally with the pure compound(s) against pathogenic mycobacteria and non-tuberculous mycobacteria. Through bioassay-guided fractionation, ten compounds active against mycobacteria were observed using bioautography but only three compounds could be isolated in sufficient quantity within the time frame allocated to the completion of this research for the degree purpose as compounds were closely related to each other and difficult to separate. Due to time constraints the three compounds were only tested against the non-tuberculous mycobacteria.

1.7 STRUCTURE OF THE THESIS

Chapter 1 provides an overview related to the general aspects of the disease caused by mycobacteria spp. and the use of plants as alternative medicine and potential leads for drug development. Epidemiology, causes, pathogenesis, clinical signs, diagnosis, control, treatment and zoonotic aspects of the diseases are covered.

Chapter 2 deals with samples from different animal species suspected of being infected with tuberculosis. Isolation of mycobacteria spp. using solid medium, Löwenstein-Jensen agar and liquid medium tubes using the BACTEC™ MGIT™ is described. Isolates were identified and characterized using Ziehl-Neelsen staining, the line probe assay from the GenoType CM/AS kit, multiplex PCR, spoligotyping, variable number tandem repeat. Antimycobacterial susceptibility testing using a commercial kit, Genotype® MTBDR*plus* against first line drugs was also conducted on some isolates. Results of mycobacteria spp. identified and antimycobacterial activity of drugs against some isolates are reported.

Chapter 3 describes the novel *Mycobacterium avium* complex isolated from black wildebeest samples suspected of tuberculosis. Isolation using solid medium, Löwenstein-Jensen slants, and liquid medium tubes using the BACTEC™ MGIT™ are reported. Identification using biochemical tests, the line probe assay from the GenoType CM/AS, gene sequencing of all four genes 16S rRNA, 16S-23S rRNA (ITS), *rpo*B and *hsp*65 and phylogenetic analyses are discussed.

Chapter 4 analyses results obtained from the screening of 15 plant species against pathogenic and rapidly growing mycobacteria. Cytotoxicity evaluation of selected plants with good antimycobacterial using a colorimetric assay against Vero monkey kidney, human hepatoma and murine macrophage cells is discussed. Results justifying the selection of *Searsia undulata* as the plant of interest for further studies are presented. An incidental finding related to the potential anticancer property of *S. undulata* is reported.

Chapter 5 provides results of the bioassay-guided fractionation of the bulk plant extract leading to the isolation of compounds. Results of antimycobacterial activity, cytotoxicity assays and structure elucidation of isolated compounds using MS and NMR spectroscopy are reported. The physio-biological properties of one of the compounds are provided.

Chapter 6 covers the highlights of results obtained from each previous chapter in a coherent manner. Assessment of each objective is discussed; challenges encountered during the course of this research are provided, and future perspectives and recommendations in terms of work subsequent to this PhD research are also provided.

CHAPTER 2:
MOLECULAR PROFILE OF *MYCOBACTERIUM* SPP. ISOLATES FROM CATTLE AND OTHER ANIMAL SPECIES

ABSTRACT

Mycobacterium bovis causes tuberculosis in cattle and other animals and its incrimination in human tuberculosis has been recognized since the beginning of the twentieth century. Tuberculosis caused by *M. bovis* or *M. tuberculosis* presents the same clinical picture. The treatment of tuberculosis is currently a challenge due to emergence of multi-drug and extensively drug resistant strains. Therefore, there is a need to characterize and establish the genetic diversity of the current isolates used in antimycobacterial bioassays while exploring the development of alternative drugs for tuberculosis. Consequently, a purposive sampling was conducted and a total number of samples (n=80) were processed for *Mycobacterium* spp. isolation that included 31 samples from 10 cattle; 26 samples from 12 black wildebeest (*Connochaetes gnou*) which were received in two different batches, 20 samples from 20 warthogs (*Phacochoerus africanus*), 1 sample from an impala (*Aepyceros melampus*) and 2 samples from 1 rabbit (*Oryctolagus cuniculus*). The isolation, multiplex PCR, spoligotyping, variable number of tandem repeat (VNTR) and antimicrobial susceptibility test of some of the isolates against first-line TB drugs using Genotype® MTBDR*plus* kit (Hain Life Science GmbH, Nehren, Germany) were investigated.

In this chapter, samples (n=64) are described, which includes the second batch of samples (n=10) from 3 black wildebeest. The findings from the first sample batch (n=16) from black wildebeest are reported in Chapter 3. The 64 samples which yielded 36 isolates of *Mycobacterium* spp. included samples (n=10) from 3 black wildebeest that yielded *M. avium* subsp*. hominissuis*, samples (n=20) from 20 warthog that yielded *M. bovis* (n=14), *M. avium* (n=1) and *M. intracellulare* (n=1), samples (n=2) from a rabbit that yielded *M. avium* subsp*. hominissuis* (n=1), sample (n=1) from 1 impala yielded *M. bovis (n=1)* and samples (n=31) from 10 cattle that yielded *M. bovis* (n=15).

The non-tuberculous mycobacteria isolated from the rabbit, black wildebeest samples and warthogs (*M. intracellulare* and *M. avium*) are potentially zoonotic as they have been incriminated in diseases in humans.

The isolates from cattle were further analysed by spoligotyping and yielded two spoligotypes. Ten out of 15 *M. bovis* isolates were typed as SB0121 (67%) and 5 out of 15 were typed as SB 1235 (33%). SB0121 was obtained from different animals and it has been isolated from several countries including South Africa whereas SB1235 seemed to relate to UK strains and was recently reported in South Africa. A spoligotype strain is an indication of the scope of the movement of cattle and it is a useful epidemiological tool. All isolates had an identical VNTR type 1 which meant that the isolates were from the same origin. The antimicrobial sensitivity test of *M bovis* isolates revealed that 8 out of 15 (53%) isolates were multi-drug resistant (MDR) as they were resistant to the first-line drugs INH and RIF used routinely in the tuberculosis regimen in humans whereas 7 out of 15 (47%) were sensitive to both drugs. The presence of MDR of *M. bovis* in cattle is of concern considering the emergence of MDR-TB strains in humans and the lack of data on the prevalence and incidence of tuberculosis caused by *M. bovis* in humans in South Africa.

2.1 INTRODUCTION

Tuberculosis (TB) is the most common cause of death in this century. In 1993, the World Health Organization (WHO) declared TB to be a global emergency. *Mycobacterium tuberculosis* and *Mycobacterium bovis* are the main causative agents associated with the disease in humans. They belong to the *Mycobacterium tuberculosis* complex (MTC) which includes other species, namely *M. africanum, M. canetti, M. pinnipedii, M. caprae, M. microti, M. mungi, Dassie bacillus, M. orygis* (*Oryx bacillus*) and *M. surricatae*. These species are pathogenic and can cause tuberculosis in mammalian hosts (Alexander *et al*., 2010; Helden *et al*., 2009; Pittius *et al*., 2012; ; Vos *et al*., 2001). In industrialized countries, human infection with *M. bovis* has been largely controlled through pasteurization of cow's milk, abattoir inspection and culling of cattle positive reactors to the compulsory tuberculin test. Despite these control measures, cases of human tuberculosis caused by *M. bovis* associated with or without HIV/AIDS have been reported (CDC, 2005; De Kantor *et* *al*., 2010; Evans *et al*., 2007; Gallivan *et al*., 2015; Hlavsa *et al*., 2008; Olea-Popelka *et al*., 2016) as well as cases of co-infection with both *M. bovis* and *M. tuberculosis* (Silva *et al*., 2013). Cases of animal tuberculosis caused by *M. tuberculosis* have also been reported (Michel *et al*., 2013; Ocepek *et al*., 2005). The incidence of tuberculosis caused by *M. bovis* in humans is underestimated in most cases due to the difficulty of differentiating *M. tuberculosis* from *M. bovis* with limited diagnostic tools such as culture medium and Ziehl-Neelsen stained smears. Adding to this, human tuberculosis caused by either *M. tuberculosis* or *M. bovis* is clinically undistinguishable (Cousins, 2001; De la Rua-Domenech, 2006; Grange, 2001). Airborne human to human transmission of *M. bovis* has been reported in the UK (Evans *et al*., 2007).

It was estimated that between 2002 and 2020, one billion people would be newly infected, 200 million people would get sick, and 35 million would die of TB if proper control measures were not instituted (WHO, 2002). The treatment of tuberculosis is still a challenge due to emergence of multi-drug and extensively resistant strains (Bateman, 2015; Hoang *et al*., 2015; WHO, 2015). Several authors have reported outbreaks of multi-drug resistant (MDR) *M. bovis* and *M. tuberculosis* strains (Bateman, 2015; Samper *et al*., 1997).

These outbreaks highlight the importance of accurate diagnosis of *Mycobacterium* spp. for effective management of the disease. Besides the solid culture medium, Löwenstein-Jensen (LJ) with glycerol that is routinely used in medical laboratories, it is recommended to include LJ with pyruvate which will promote the growth of *M. bovis* and prevent missing cases of *M. bovis* (Silva *et al*., 2013). Beyond culture isolation and Ziehl-Neelsen staining, other methods of differentiation are used to identify *Mycobacterium* spp. namely MIRU-VNTR (Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeat), Restriction Fragment Length Polymorphism (RFLP) typing, gene sequencing, spoligotyping and antimicrobial susceptibility (Laniado-Laborín *et al*., 2014; Michel *et al*., 2013; Ocepek *et al*., 2005). Antimicrobial sensitivity profile can assist in the differentiation between *M. tuberculosis* and *M. bovis* while providing the susceptibility pattern against antimycobacterial drugs for an effective drug choice. For example *M. bovis* is known to be resistant to pyrazinamide; such isolates should further be analysed to rule out *M. tuberculosis*. A combination of techniques is required for an accurate identification of the isolates. Spoligotyping, RFLP typing, gene sequencing and VNTR are important as epidemiological tools to trace the origin of the infection, identify the strain involved and assist in disease control especially in eradication programmes aiming at eliminating the sources of infection (Hang'ombe *et al*., 2012; Hlokwe *et al*., 2013; Michel *et al*., 2013).

Other challenges include adverse drug side effects, high loss of follow ups of patients (suboptimal treatment adherence), inadequate treatment of MDR-TB using fewer drugs, leading to XDR-TB, inadequate hospital capacity, poor infection control and limited availability of appropriate second-line drugs and inadequate national funds from government (Bateman, 2015; Hoang *et al*., 2015; WHO, 2015). South Africa is ranked among the 30 countries with a high burden of multi-drug resistant tuberculosis (Churchyard *et al*., 2014; WHO, 2015). The current antimycobacterial drugs include isoniazid (INH) and rifampin (RIF) as most common first-line drugs and fluoroquinolones as second-line drugs; patients diagnosed with MDR-TB do not respond to the treatment with first-line drugs (INH and RIF) and therefore they will have to be treated with second-line drugs. The current recommendation from WHO for MDR and XDR is a minimum of 20 months therapy using 3 or 4 drugs in combination (WHO, 2015) but lower cure rates of MDR and XDR-TB have been observed, ranging from 50 to 70% which have led to high morbidity and mortality rates (Gandhi *et al*., 2012).

In the absence of a preventative vaccine, more effective diagnostic tools and novel therapeutic interventions are needed. An understanding of the relationship between strains and resistance, virulence and drug resistance, is necessary to fully understand the current disaster as well as needs to identify alternative treatments besides current available drugs (WHO, 2010b).

There are different approaches to explore alternative treatment for TB namely, modification of current molecules of drugs already on the market and exploration of natural resources such as plants (Jimenez-Arellanes *et al*., 2003; Newman *et al*., 2003). Plants have been the basis of many traditional medicine systems throughout the world for centuries and continue to provide mankind with new remedies. Besides synthetic chemistry, plants are still an important natural source of pharmacologically active compounds with many drugs derived directly or indirectly from plants (Newman *et al*., 2003; Veeresham, 2012).

When conducting the different bioassays exploring alternative treatments for tuberculosis, it is relevant to make use of new isolates of *Mycobacterium* spp. from the changing environment as well as reference cultures sourced for example from the American Type Culture Collection (ATCC) strains and National Collection of Type Cultures (NCTC). Therefore, there is a need to characterize and establish the genetic diversity of the current isolates that can be used in bioassays exploring the development of alternative drugs for tuberculosis. This will also allow detection of any changes in circulating strains and epidemiological traceability of *Mycobacterium* spp. causing disease.

2.2 MATERIALS AND METHODS

2.2.1 Study area

In South Africa, nine provinces exist but due to the vast land mass of the country, it was necessary to select some study areas based on reported cases and accessibility to samples within the time frame allocated to this research. Mpumalanga Province was selected as the area of study based on previous publications reporting TB in wildlife and livestock (Bengis *et al*., 1996; Michel *et al*., 2007; Vos *et al*., 2001) and availability of veterinary staff members experienced in conducting the comparative tuberculin skin test. Mpumalanga is one of the nine provinces in South Africa as indicated by a red triangle on the map (Figure 2.1) and it is located in the north-eastern part of the country, bordering Swaziland and Mozambique to the East. It embraces the southern half of the Kruger National Park, a vast nature reserve with abundant wildlife including big game. It has a subtropical foliage supporting about 1 439 000 cattle according to the Trends in the Agricultural Sector (2013).

Figure 2.1: South Africa Map with the red triangle showing Mpumalanga Province where samples were obtained

Source: courtesy of www.sa.venues.com

2.2.2 Study design and sampling

The study was designed as a cross sectional study and specimens were collected from animals in the designated areas from January 2009 to January 2011. The local municipalities were selected based on the number of commercial farms, proximity to abattoirs, and location at the human-wildlife interface and also the movement of animals. The municipalities selected comprised Malelane, Nelspruit, Lydenburg, Ermelo, Witbank and Standerton. The target population comprised cattle carcasses showing gross tuberculous-like lesions at meat inspection from positive reactors to tuberculin skin test at the municipality abattoirs during the study period. Samples from any other animal species showing gross tuberculous-like lesions were also included as convenience samples. The sampling was purposive to increase the chances of isolating mycobacteria and only cattle were selected based on positive reaction on tuberculin skin test and pathological lesions.

2.2.3 Sources of Samples

Thirty-one samples randomly selected from 10 cattle (*Bos taurus*) from 3 different farms and one abattoir located in Mpumalanga were screened for *Mycobacterium* spp. isolation and identification. This work was conducted at the National Health Laboratory Service (NHLS) in Pretoria, South Africa. Samples from other animal species were collected based on observed tuberculosis-like lesions. They comprised two samples from a rabbit (*Oryctolagus cuniculus*), 20 samples from 20 warthogs (*Phacochoerus africanus*), a different batch of 10 samples from 3 black wildebeest and 1 sample from an impala (*Aepyceros melampus)*. Different organs and lymph nodes on ice were received (Table 2.1).

All samples were accompanied by a sample submission form that contained information about the animal, owner and location. The history of each animal was provided by the state veterinarian in charge. Cattle samples were from positive reactors to tuberculin test and that had lesions suggestive of tuberculosis at slaughter. Yellowish granulomatous lesions of different sizes with calcification and gritty sensation when cutting through the granulomas were observed.

Table 2.1: Types of samples from different animal species

	Tissue samples													Total
	Colon	Liver	Lung	Kidney	Br	Ext. Il	Ing	Hep	Med	Par	Pres	Retro	Mand	
Animal species														
Black wildebeest (n=3)	0	0	0	0	5	0	0	0	5	0	0	0	0	10
Cattle (n=10)	0	2	2	2	10	2	2	0	2	3	2	2	2	31
Impala (n=1)	0	0	0	0	1	0	0	0	0	0	0	0	0	1
Rabbit (n=1)	1	0	0	0	0	1	0	0	0	0	0	0	0	2
Warthog (n=20)	0	0	0	0	5	0	0	2	0	3	0	5	5	20
Total per category	1	2	2	2	16	3	2	2	2	6	2	7	7	64

Br: bronchial; Ex IL: External iliac; Ing: Inguinal; Hep: Hepatic; Med: Mediastinal; Par: Parotid; Pres: Prescapular; Retro: Retropharyngeal; Man: Mandibular

2.2.4 Mycobacterial isolation

Samples were kept frozen at -20°C until processing at NHLS under biosafety measures (Figure 2.2). Direct impression smears were made from lesions and were stained using the Ziehl-Neelsen method. Tissue samples taken in a sterile manner from the inside of granulomatous lesions at the border between healthy and fibrous tissues were finely cut using a sterile scalpel blade and homogenized using sterile glass beads by vortexing as described by Bengis *et al*. (1996) and Warren *et al*. (2006) with some modifications. To maximize the mycobacterial yield, specimens were subjected to a digestion and decontamination procedure using N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) solution with NaOH at a final concentration of 2% (Chatterjee *et al.*, 2013). The specimens were left at room temperature for 15 min during the decontamination process and thereafter neutralized with phosphate buffer pH 6.8, centrifuged (Beckman Coulter) at 3000 x g for 15 min at 4°C, and the supernatant decanted and pellet suspended into 1 mL of phosphate buffer. The sediment was inoculated onto two LJ slants, supplemented with pyruvate and glycerol and an antibiotic mixture of polymyxin B, amphotericin B, carbenicillin and trimethoprim (PACT) (National Health Laboratory Service, South Africa, and Becton Dickinson, Germany) using a 0.01 mL calibrated loop. A further 0.5 mL of the sediment was inoculated with a graduated Pasteur pipette into a prepared liquid medium tube (BBL^{TM} MGIT^{TM} Mycobacterium Growth indicator tubes) enriched with OADC and containing 800 µl of PANTA^{TM} antibiotic mixture (BD^{TM}). This was incubated in the BACTEC^{TM} MGIT^{TM} 960 (Figure 2.2) mycobacterial detection system at 37°C (Warren *et al.*, 2006). The system was monitored for a maximum period of 42 days for bacterial growth whereas LJ slants were observed for colony growth and any other contaminants at two week intervals for 10 weeks. Tubes detected as positive within that period were further processed using Ziehl-Neelsen staining and examined microscopically for the presence of acid-fast organisms and morphology, thereafter subcultured on LJ slant supplemented with glycerol and pyruvate. For identification purposes, a single colony was subcultured on a fresh LJ slant to obtain pure colonies. The same was repeated with two other colonies on different LJ slants to rule out the possibility of missing a different organism. Reference cultures of *M. avium* (ATCC 25291), *M. bovis* BCG and *M. tuberculosis* (ATCC 25177) were used as positive controls. Stored DNA extracts were preserved in a final concentration of 1 mM EDTA 24 hours prior to being taken to Japan. Disodium salts dehydrate, EMD Biosciences, inc. La Jolla, CA 92039-2087 was used as a preservative.

(a) (b)

Figure 2.2: Samples processed in a biosafety cabinet (a) and incubated in BACTEC™ MGIT™ 960 system (b)

2.2.5 Mycobacterial identification

2.2.5.1 DNA extraction and primary molecular identification

DNA extracts were prepared based on the GenoType CM/AS reverse line blot assay (Hain Life Science, Gmbh Nehren, Germany). DNA was extracted from colonies on LJ slants by heating at 95°C for 20 min in a water bath. Primers provided by the manufacturer's kit (Hain Lifescience, Nehren, Germany) were used. The formula for the PCR assay per tube mixture contained 1.1 µL of ultra-pure water, 5 µL of 10 x buffer, 3.6 µL of $MgCl_2$ (25 mM), 35 µL of primer/nucleotide mix from the kit and 0.3 µL of hot Taq polymerase. The thermocycler was programmed to initiate the PCR as follows: one cycle of 15 min at 95°C, followed by 10 cycles of 30 sec at 95°C, 2 min at 58°C, 25 sec at 95°C; then 20 cycles of 40 sec at 53°C and 40 sec at 70°C with a final cycle of 8 min at 70°C. The hybridization was then followed as per manufacturer's instructions using the strips provided in the kit (DNA strip

Mycobacterium identification species). Part of each DNA extract was also stored at -20°C prior to further investigations in Japan.

2.2.5.2 Antimicrobial susceptibility test

The antimicrobial susceptibility test and identification of mutations responsible for resistance against rifampin (RIF) and isoniazid (INH) were conducted at NHLS according to in house standard operating procedure using a commercial kit, Genotype® MTBDR*plus* kit (Hain Life Science, Gmbh Nehren, Germany), following the manufacturer's instructions. Biotinylated primers provided with the kit were used to amplify 5 µl of DNA with hot-start Taq DNA polymerase (Qiagen, Pretoria, South Africa). Amplification was performed using a thermal cycler MyCycler™ (Bio-Rad, Cape Town, South Africa). The thermocycler was programmed as described under section 2.2.5.1. Then hybridization steps were followed using hybridization trays (Hain Life Science, Gmbh Nehren, Germany) according to the manufacturer's instructions. The following genes were targeted for detecting the sensitivity of the isolates to a specific antimycobacterial drug namely eight *rpo*B wild-type probes named WT1-WT8 and four mutant probes named MUT1, MUT2A, MUT2B and MUT3 were used to detect resistance against RIF. One *kat*G wild type (*kat*G WT) and two mutant probes (MUT1 and MUT2); plus two *inh*A wild type (WT1 and WT2) and four mutant probes (MUT1, MUT2, MUT3A and MUT3B) for the detection of resistance against INH. According to the manufacturer, when all WT probes stained positive and no mutation band formed, the result was interpreted as susceptible to the specific anti-TB drug (Figure 2.3). The resistance towards a specific antibiotic was indicated by the absence of a band for at least one of the WT probes. Each pattern deviating from the wild type pattern indicates a resistance of the tested strain. The banding pattern obtained with rpoB probes allows a conclusion to be drawn about a rifampicin resistance of the strain tested whereas the banding pattern obtained with the *kat*G probes allows a conclusion to be made about a high level isoniazid resistance; the banding pattern obtained with the *inh*A probes allows a conclusion to be drawn about a low level of isoniazid resistance of the strain tested respectively.

2.2.5.3 MTC discrimination by multiplex PCR

The *Mycobacterium tuberculosis* complex-discriminating multiplex PCR (MTCD-MPCR) targeting genetic regions cfp32 (a specific gene for MTC), RD4 (region of difference 4 which is present in *M. tuberculosis* and *M. africanum* but absent in *M. bovis* was used for species differentiation according to the method described by (Parsons *et al*., 2002) with slight modifications. The reaction mixture consisted of 7.4 µL H_20, 2 µL 10 x Taq buffer, 2 µL dNTPs (2.5 mM each), 0.2 µL Taq (Takara Bio Inc, Shiga, Japan), 1 µL of DNA sample, 2.2 µL of 10 µM cfp32 primers, 0.7 µL of 5 µM RD4 primers. The PCR was performed using the following program: denaturation for 1 min at 98°C followed by 35 cycles of 5 sec at 98°C, 20 sec at 58°C and 1 min at 68°C with final elongation at 72°C for 5 min in a thermal cycler (iCycler, Bio-Rad Laboratories Inc., Hercules, CA). *Mycobacterium bovis* BCG Pasteur vaccine, *M. bovis* BCG Tokyo and *M. bovis* AF2122/97 were used as positive controls and distilled water as negative control. The PCR products were separated by electrophoresis on a 2% agarose gel in TAE buffer, and then visualized after staining with ethidium bromide. RD4 Flank F and RD4 flank R gave a product of about 446 bp.

2.2.5.4 Spoligotyping

Spoligotyping of *M. bovis* isolates using microarray was performed according to the procedure by Kamerbeek *et al*. (1997) with slight modifications. Forty-three spacer-sequence probes were covalently bound to the membrane (Pall Co., NY, USA). PCR products were generated using primers DRa (GGTTTTGGGTCTGACGAC) and DRb (CCGAGAGGGGACGGAAAC). The PCR mixture included 7.3 µL of deionized water, 5 X 3 µL colourless GoTaq buffer (Promega TM, Fitchburg, WI), 1.5 µL of PCR DIG Labelling Mix (Roche), 1 µL of 10 µM Fw primer, 11 µL of 10 µM Rv primer, 0.2 µL of GoTaq DNA Polymerase (5 units/µL, Promega) and 1 µL of DNA sample in a total volume of 15 µL of mixture per tube. The thermocycler was programmed to initiate denaturation at 98°C for 60 sec, followed by 40 cycles of 5 sec at 98°C, 10 sec at 55°C, 10 sec at 55°C, 30 sec at 72°C and a post run for 60 sec at 72°C. The PCR product that was diluted 500 times in hybridization buffer was heat denatured at 95°C for 5 min and immediately cooled on ice to obtain the single stranded DNA. The probe solutions for spotting were prepared by adjusting the concentration with TE buffer to 50 µM of spacer probe and 10 µM of DR probe (positive control). Then the membrane was cut to an appropriate size and marked to distinguish the side to be spotted. The membrane was held with forceps to avoid binding of protein, which could interfere with probe DNA attachment on the membrane. Then 0.5 µL of each probe solution was spotted on the membrane and heated at 70°C for 30 min, followed by pouring of the blocking buffer on the membrane. It was then allowed to stand at room temperature for 1 hour with intermittent gentle shaking.

The hybridization was carried out by immersing the membrane in the hybridization solution at 60°C for 1 hour. Then, the membrane was washed in warm TBST-E (0.1% Tween-20 and 1 mM EDTA-2Na in tris-buffered saline) and incubated for 10 min at 60°C then washed for the third time in warm TBST-E. The membrane was then dried at room temperature. The POD and TMB solutions were prepared as per manufacturer's instructions. The nitrocellulose membranes were covered with a 1000 times diluted Ant-Digoxigenin-POD [poly], Fab fragments (Roche) in a dish and incubated at room temperature for 30 min. Thereafter, the membranes were sequentially washed three times in TBST-E at room temperature for 1 min, 10 min and 1 min as per protocol (Hang'ombe *et al*., 2012). Then, POD on the membranes was detected through colour development by adding TMB solution (TMB Peroxidase Substrate Kit, Vector Labs Inc. TM, Burlingame, CA) according to the manufacturer's protocol. The membrane was then washed with deionized water (DIW) and scanned (Figure 2.4). The spoligotype patterns observed were compared to the patterns available in the spoligotype *M. bovis* database (www.mbovis.org)

2.2.5.5 Variable number of tandem repeat (VNTR) typing

The *M. bovis* isolates were further genotyped by PCR amplification using primers targeting a wide range of tandem repeat loci (Table 2.2). Two different PCR reaction mixtures were conducted according to the loci and the PCR primer sequences used are outlined in Table 2.2. Locus MIRU10, 16, 24, 26, 27, 39, 40, ETR-B, F, VNTR-424, 3690 were done under betaine 1.0 M, whilst locus MIRU2, 4, 20, 23, 31, ETR-A, C, QUB- 11a, 11b, 26, 3232, 3336, VNTR-1955, 2401, 4156 were performed under GCII buffer (Takara). The PCR reaction mixture under 1.0 M betaine buffer was conducted in a mixture consisted of 6.3 µL H2O, 0.4 µL of dNTP (10 mM each), 3.0 µL of 5x colourless Go Taq buffer, 3.0 µL of betaine (5 M), 0.6 µL of Primer 1 and 0.6

μL of Primer 2, 0.1 μL of Go Taq (5 units/ μL) DNA Polymerase, and 1 μL of DNA sample. An initial denaturation step of 95°C for 5 min was followed by 32 cycles at 95°C for 15 sec, 58°C for 20 sec and 72°C for 1 min with a final elongation step at 72°C for 1 min in a thermal cycler. The PCR reaction mixture under the GCII buffer consisted of 4.8 μL H_2O, 0.4 μL of dNTP (10 mM each), 7.5 μL of 2 x GCII buffer, 0.6 μL of primers, 0.1 μL of Go Taq (5 units/μL) DNA Polymerase (Promega), and 1 μL of DNA sample. All the PCR samples were electrophoresed on a 2% agarose gel to identify the repeat numbers; a 100 bp ladder PLUS (Thermo Scientific, USA) was included for the estimation of the size (Hang'ombe *et al*., 2012) (Table 2.3).

Table 2.2: Variable number of tandem repeat (VNTR) loci and forward and reverse primers (5'-3') sequences used for the typing of *M. bovis* isolates (Le Fleche *et al*., 2002)

ON-No	Locus No	Common names	Primer sequence	Mer
749	13	MIRU4F	GCGCGAGAGCCCGAACTGC	19
750		MIRU4R	GCGCGAGCAGAAACGCCAGC	20
751	1	MIRU10F	GTTCTTGACCAACTGCAGTCGTCC	24
752		MIRU10R	GCCACCTTGGTGATCAGCTACCT	23
753	2	MIRU16F	TCGGTGATCGGGTCCAGTCCAAGT	24
754		MIRU16R	CCCGTCGTGCAGCCCTGGTAC	21
755	3	MIRU26F	CCCGCCTTCGAAACGTCGCT	20
756		MIRU26R	TGACATAGGCGACCAGGCGAATA	24
757	4	MIRU31F	ACTGATTGGCTTCATACGGCTTTA	24
758		MIRU31R	GTGCCGACGTGGTCTTGAT	19
759	5	MIRU40F	GGGTTGCTGGATGACAACGTGT	22
760		MIRU40R	GGGTGATCTCGGCGAAATCAGATA	24
761	9	ETR-A1	AAATCGGTCCCATCACCTTCTTAT	24
762		ETR-A2	CGAAGCCTGGGGTGCCCGCGATTT	24
763	10	ETR-C1	GTGAGTCGCTGCAGAACCTGCAG	23
764		ETR-C2	GGCGTCTTGACCTCCACGAGTG	22
765	11	QUB11b1	CGTAAGGGGGATGCGGGAAATAGG	24
766		QUB11b2	CGAAGTGAATGGTGGCAT	18
767	12	QUB26-1	GGCCAGGTCCTTCCCGAT	18
768		QUB26-2	AACGCTCAGCTGTCGGAT	18
769	6	VN424F	CTTGGCCGGCATCAAGCGCATTATT	25
770		VN424R	GGCAGCAGAGCCCGGGATTCTTC	23
771	14	VN2401F	CTTGAAGCCCCGGTCTCATCTGT	23
772		VN2401R	ACTTGAACCCCCACGCCCATTAGTA	25
773	8	VN3690F	CGGTGGAGGCGATGAACGTCTTC	23
774		VN3690R	TAGAGCGGCACGGGGGAAAGCTTAG	25
775	15	VN4156F	TGACCACGGATTGCTCTAGT	20
776		VN4156R	GCCGGCGTCCATGTT	15

777	7	VN1955F	AGATCCCAGTTGTCGTCGTC	20
778		VN1955R	CAACATCGCCTGGTTCTGTA	20
779	16	MIRU2F	TGGACTTGCAGCAATGGACCAACT	24
780		MIRU2R	TACTCGGACGCCGGCTCAAAAT	22
781	17	MIRU20F	TCGGAGAGATGCCCTTCGAGTTAG	24
782		MIRU20R	GGAGACCGCGACCAGGTACTTGTA	24
783	18	MIRU23F	CAGCGAAACGAACTGTGCTATCAC	24
784		MIRU23R	CGTGTCCGAGCAGAAAAGGGTAT	23
785	19	MIRU24F	CGACCAAGATGTGCAGGAATACAT	24
786		MIRU24R	GGGCGAGTTGAGCTCACAGAA	21
787	20	MIRU27F	TCGAAAGCCTCTGCGTGCCAGTAA	24
788		MIRU27R	GCGATGTGAGCGTGCCACTCAA	22
789	21	MIRU39F	CGCATCGACAAACTGGAGCCAAAC	24
790		MIRU39R	CGGAAACGTCTACGCCCCACACAT	24
791	22	ETR-B1	GCGAACACCAGGACAGCATCATG	23
792		ETR-B2	GGCATGCCGGTGATCGAGTGG	21
793	23	ETR-F1	CTCGGTGATGGTCCGGCCGGTCAC	24
794		ETR-F2	GGAAGTGCTCGACAACGCCATGCC	24
795	24	QUB11a1	TTCAGGGGGGATCCGGGA	18
796		QUB11a2	CCCATCCCGCTTAGCACATTCGTA	24
797	25	QUB3232-1	CAGACCCGGCGTCATCAAC	19
798		QUB3232-2	CCAAGGGCGGCATTGTGTT	19
799	26	QUB3336-1	ATCCCCGCGGTACCCATC	18
800		QUB3336-2	GCCAGCGGTGTCGACTATCC	20

Table 2.3: Band sizes (bp) and corresponding tandem repeat numbers

			Tandem repeat number for M. bovis										
No	Locus name	Unit (bp)	0	1	2	3	4	5	6	7	8	9	10
1	MIRU10	53	482	537	590	643	696	749	802	855	908		
2	MIRU16	53	565	618	671	724	777	830	883	936	989		
3	MIRU26	51	461	512	563	614	665	716	767	818	869		
4	MIRU31 (ETR-E)	53	492	545	598	651	704	757	810	863	916		
5	MIRU40	54	354	408	462	516	570	624	678	732	786		
6	VN424 (Mtub04)	51	538	589	640	691	742	793	844	895	946		
7	VN1955 (Mtub21)	57	149	206	263	320	377	434	491	548	605		
8	VN3690 (Mtub39)	58		330	388	446	504	562	620	678	736		
9	ETR-A(VN2165)	75	195	270	345	420	495	570	645	720	795	870	945
10	ETR-C(VN577)	58	44	102	160	218	276	334	392	450	508		
11	QUB11b(VN2163b)	69		136	205	274	343	412	481	550	619		
12	QUB26(VN4052)	111		264	375	486	597	708	819	930	1041		
13	MIRU04 (ETR-D)	77 or 53		252	329	406	483	560	637				
14	VN2401 (Mtub30)	58	247	305	363	421	479	537	595	653	711		
15	VN4156n(QUB4156)	59		563	622	681	740	799	858	917	976		
16	MIRU02	53	402	455	508	561	614	667	720	773	826		
17	MIRU20	77		514	591	668							
18	MIRU23	53	558	608	661	714	767	820	873	926	979		
19	MIRU24	54	395	447	501	555	609	663	717	771	825		
20	MIRU27	53		551	604	657	710	763	816	869	922		
21	MIRU39	53	536	591	646	701	756	811	866	921	976		
22	ETR-B	57	121	178	235	292	349	406	463	520	577		
23	ETR-F	79	263	342	421	500	579	658	737	816	895		
24	QUB11a(VN2163a)	69		374	443	512	581	650	719	788	857	926	995
25	QUB3232	56	423	479	535	591	647	703	759	815	871		
26	QUB3336	59		171	230	289	348	407	466	525	584	643	702

2.3 RESULTS AND DISCUSSION

2.3.1 Mycobacterial isolation

Out of the 20 samples received from 20 warthogs, 16 different types of lymph nodes namely bronchial, mandibular, parotid and retropharyngeal yielded either rough or smooth colonies on LJ slants with pyruvate inoculated with the sediment from positive MGIT tubes. One out of the 2 samples (external iliac lymph node) from a rabbit yielded smooth colonies as well as 3 out of 10 samples from 3 black wildebeest, 15 samples out of 31 samples from 10 cattle yielded rough colonies. All isolates were slow-growing organisms and acid-fast rods under the microscope. Isolates were further identified as Ziehl-Neelsen staining alone cannot differentiate *Mycobacterium* species. In medical laboratories, it was observed that LJ with glycerol is routinely used; introducing LJ with pyruvate in the routine diagnostic will increase the opportunity to isolate *M. bovis.*

2.3.2 Primary molecular identification

The isolates from 3 black wildebeests were identified as *M. avium* subsp*. hominissuis* (n=*3)* out of the 10 samples; 16 isolates from the 20 warthogs were identified as *M. bovis* (n=14), *M. avium* (n=1) and *M. intracellulare* (n=1), one isolate from rabbit (n=1) was also identified as *M. avium* subsp*. hominissuis,* the isolate from an impala (n=1) was identified as *M. bovis* and 15 isolates from 10 cattle were identified as *M. bovis* (Table 2.4) using commercial kits, the GenoType Mycobacterium CM/AS (Hain Lifescience GmbH, Nehren, Germany). Cattle isolates were further processed as species of interest for the next phase of the research project whereas those from other animal species were stored at -80°C for use in future studies.

The isolation of non-tuberculous mycobacteria namely *M. avium* subsp*. hominissuis*, *M. avium* and *M. intracellulare* are of concern as these species are potentially zoonotic and have been reported as causes of disease in humans. In South Africa, the level of involvement of non-tuberculous mycobacteria in disease-causing conditions in animals and humans should be fully investigated. In the medical laboratory where the study was conducted, these mycobacteria were referred as “MOTT” and are not given much attention.

Table 2.4: *Mycobacterium* species isolated from the different animals

Animal species (n=5)	*Mycobacterium* spp.
Black Wildebeest	*M. avium* subsp. *hominissuis*
Cattle	*M. bovis*
Impala	*M. bovis*
Rabbit	*M. avium* subsp. *hominissuis*
Warthog	*M. bovis, M. intracellulare* and *M. avium*

2.3.3 MTC discrimination by multiplex PCR

Out of 31 samples, 15 isolates were primarily identified as *M. bovis* using the GenoType Mycobacterium CM/AS (Hain Lifescience). They were all confirmed by MTC-MPCR as belonging to the *M. tuberculosis* complex and as *M. bovis* by the absence of RD4.

2.3.4 Antimicrobial susceptibility test

Eight out of 15 (53%) were resistant to INH and RIF whereas 7 out the 15 isolates (47%) of *M. bovis* were sensitive to INH and RIF. Figure 2.3 shows the appearance of bands as sensitivity to the drugs and absence of bands as resistance to the specific drug. None of the INH and RIF resistant samples harboured mutations in the *katG* gene or the *Inh*A gene. In South Africa, Silaigwana *et al*. (2012) reported on a similar study and found the presence of MDR-TB isolates on a dairy farm. More studies to assess the prevalence of *M. bovis* MDR-TB strains are needed because of public health implications of TB caused by *M. bovis* in both humans and animals. *Mycobacterium bovis* is known to be resistant to pyraminazide and have shown different susceptibility patterns to anti-TB drugs (Bobadilla-del Valle *et al*., 2015). These results have a clinical and epidemiological significance and further studies focusing on genes coding for resistance should be investigated.

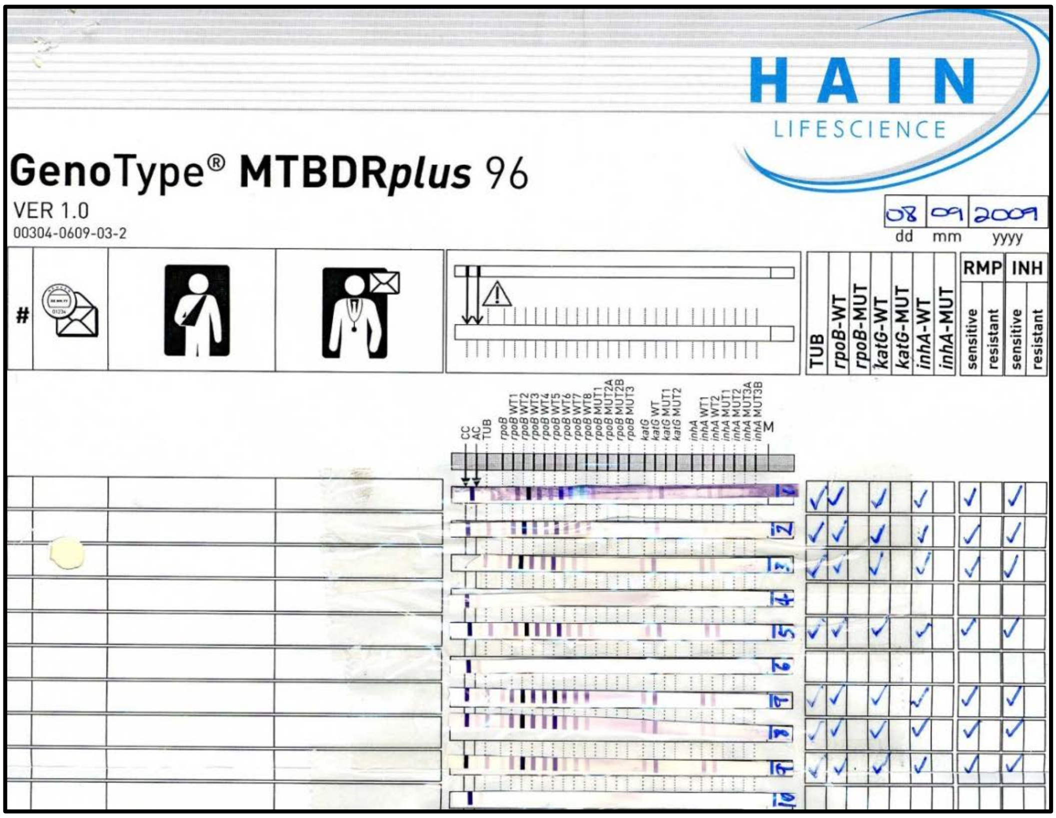


Figure 2.3: WT probes and mutation genes targeted to determine the susceptibility of an isolate towards first line drugs

2.3.5 Spoligotyping

The 15 isolates of *M. bovis* from cattle consisted of two strains, namely SB0121 and SB1235. Most isolates, 10 out of 15 were SB0121 (67 %) and 5 out of 15 (33%) were SB1235. SB1235 lacked spacers 3, 6, 8, 9, 10, 11, 12, 16, 37-43 (Figure 2.4 A) whereas SB0121 was characterized by lack of spacers 3, 9, 16, 21, 39-43 (Figure 2.4 B). SB0121 is the most frequently isolated spoligotype. Spoligotyping is used as an epidemiological tool to trace the origin of the strain causing the disease and hence can facilitate implementation of appropriate control measures. A consistent characteristic of *M. bovis* is the lack of spacers 39-43 (Figures 2.4 and 2.5).

Isolates from cattle were confirmed and further characterized using spoligotyping and VNTR. The spoligotype patterns revealed the presence of a strain recently reported in South Africa, SB1235 (Hlokwe *et al*., 2014), whereas SB0121 is known to be the most common type which has been reported in several other countries, namely Argentina, Belgium, Brazil, Spain, Great Britain, Italy, Mexico, Sweden, USA and South Africa (Hlokwe *et al*., 2014; Musoke *et al*., 2015; Parreiras *et al*., 2012; Rodriguez-Campos *et al*., 2013). They were all VNTR type 1 which means they have the same origin. Samples were from one province, Mpumalanga, although from different farms.

SB1235 seems to relate to UK strains, and the importation by breeders of cattle from Europe could explain the introduction of this strain (http://www.sussex.co.za; http://www.afrikanerbees.com). Spoligotype strains are an indication of how wide is the movement of cattle worldwide. SB1235 was originally isolated in Northern Ireland whereas SB0121 seems to be the most common spoligotype in South Africa as reported by Hlokwe *et al*., (2014). It has also been isolated worldwide from Argentina, Belgium, Brazil, Spain, Great Britain, Italy, Mexico, Sweden, USA, New Zealand and Sweden (Rodriguez-Campos *et al*., 2013; Sahraoui *et al*., 2009).

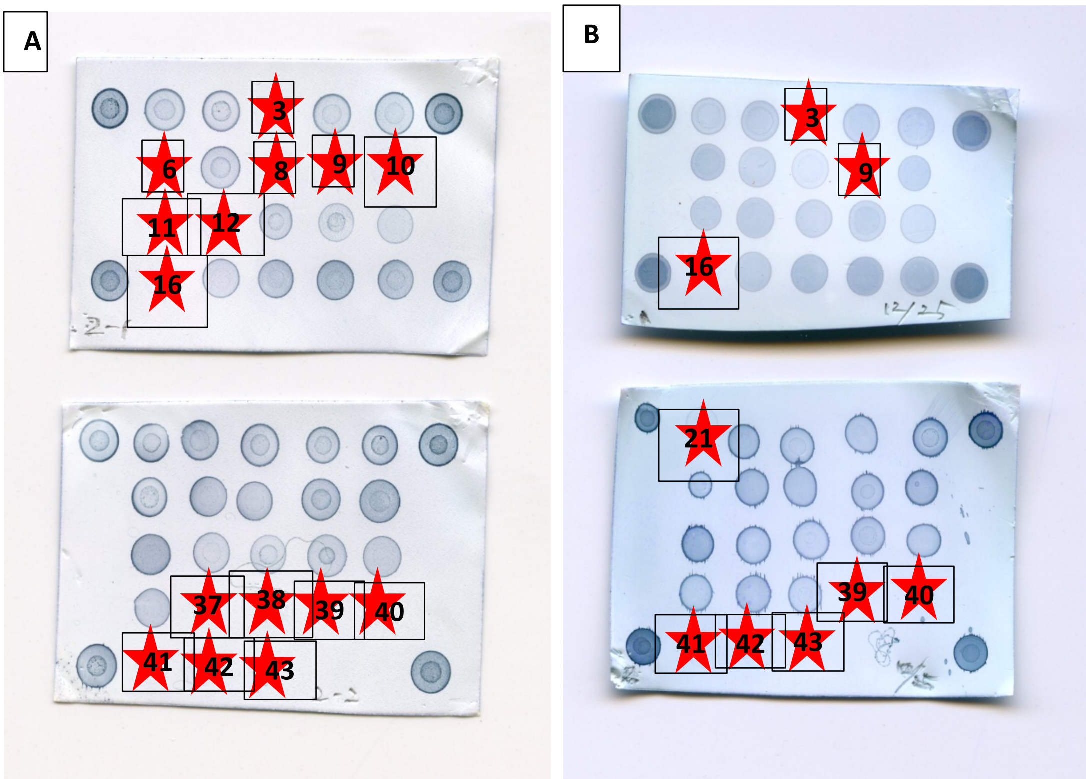


Figure 2.4: Spoligotype SB1235 (A) and spoligotype SB0121 (B), deleted spacers that are common to all *M. bovis* are 39 to 43. SB1235 lack spacers 3, 6, 8, 9, 10, 11, 12, 16, 37-43 whereas SB0121 lacks spacers 3, 9, 16, 21, 39-43 –The deleted spacers are marked with red stars

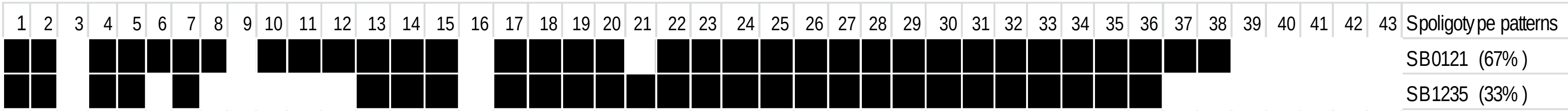


Figure 2.5: Schematic representation of spoligotype patterns of *M. bovis* cattle isolates from South Africa

2.3.6 Variable number of tandem repeat (VNTR) typing

The typing of the 15 isolates was based on the 13 VNTR loci and yielded only one type, VNTR-1. This meant that the isolates were all from the same origin.

2.4 CONCLUSION

The presence of MDR in *M. bovis* in cattle is of concern taking into consideration the emergence of MDR tuberculosis strains in humans. In medical laboratories, the tendency is to rule out *M. tuberculosis*, other mycobacteria are considered as MOTT and not given much attention. Introducing LJ with pyruvate in the routine diagnostic will increase the opportunity to isolate *M. bovis.* Attention should also be given to the non-tuberculous mycobacteria referred to as MOTT as they have been reported as the main cause of the disease in some cases. The identification of MDR-*M. bovis* isolates is of concern due to public health implications. *Mycobacterium bovis* causes tuberculosis in humans which is clinically undistinguishable from *M. tuberculosis*. The presence of MDR-MTB complex in dairy cattle in South Africa has been reported by Silaigwana *et al*. (2012). *Mycobacterium bovis* is known to be resistant to pyraminazide and has shown different susceptibility patterns to anti-TB drugs (Bobadilla-del Valle *et al*., 2015). Drug susceptibility and accurate identification of isolates in both animals and humans need attention as there are no current data on the prevalence and incidence of human tuberculosis caused by *M. bovis* in South Africa (Bobadilla-del Valle *et al*., 2015).

Genetic typing of *M. bovis* has contributed greatly to the improved knowledge of inter-bovine and interspecies transmission of bovine tuberculosis (Durr *et al*., 2000). The understanding of genetic typing is important for the effective management of bovine tuberculosis control schemes and the wildlife–livestock interface in countries where wildlife reservoirs for *M. bovis* have been identified, including South Africa (Haddad *et al*., 2004; Vos *et al*., 2001). The most widely used DNA typing techniques for *M. bovis* include IS6110 and PGRS restriction fragment length polymorphism (RFLP) typing, spoligotyping and variable number of tandem repeat (VNTR) typing (Reddington *et al*., 2011). In South Africa, spoligotyping and variable number of tandem repeat typing have been used (Hlokwe *et al*., 2014; Michel *et al*., 2007). The two methods had a high discriminatory power of over 90% (Kremer *et al*., 2005).

Another finding relates to non-tuberculous mycobacteria. Their importance has been emphasized during the last decade and it was interesting to isolate *M. avium* subsp*. hominissuis* (MAH) from the rabbit, pig samples and other samples from black wildebeest. This is the most prevalent MAC involved in human infection. Rónai *et al*. (2016) reported that *M. avium* subsp*. hominissuis* was an important member of the *M. avium* complex with a high diversity among *M. avium* subsp*. avium* and *hominissuis* and they are both potentially zoonotic. The evidence for MAH as causative agent of lymphadenitis and sometimes disseminated granulomatous inflammation in animals is supportive of virulence of the organism (Despierres *et al*., 2012). This will need some attention as *M. avium* infection is a severe condition in humans whereas in pigs it causes a subclinical infection. MAH has been isolated from swine gastrointestinal samples, from lymph nodes of tuberculin positive swine (Agdestein *et al*., 2012, 2011; Kriz *et al*., 2014; Yoav, 2012). It has also been incriminated as a cause of mesenteric lymphadenitis and sometimes disseminated granulomatous lesions in different species namely pigs, cattle, dogs and parrots (Campora *et al*., 2011; Kriz *et al*., 2014).

Arrazuria *et al*. (2015) stated that rabbits are susceptible to infection by different species of the genus *Mycobacterium,* namely *Mycobacterium avium* subsp*. avium* and *Mycobacterium avium* subsp*. paratuberculosis* but microbiological data on rabbit meat is lacking and more specifically reports of mycobacterial presence in industrially reared rabbit for human consumption have not been published.

In this research, colonic granulomatous lesions from a rabbit yielded colonies that were characterized as *M. avium* subsp*. hominissuis* (MAH) and this species is the most prevalent subtype among human MAC infection. A recent study has reported on the presence of MAH in rabbit (Arrazuria *et al*., 2015). This isolate has been stored for further investigation.

The isolates from pigs were not further characterized but have been stored for other studies especially *M. intracellulare* and *M. avium* isolates.

Some of the isolates will be used in testing of plants for antimycobacterial activity in other chapters.

Post script

The next chapter (Chapter 3) has been published in the Journal Transboundary and Emerging Diseases.

CHAPTER 3:

NOVEL *MYCOBACTERIUM AVIUM* SPECIES ISOLATED FROM BLACK WILDEBEEST (*CONNOCHAETES GNOU*) IN SOUTH AFRICA

ABSTRACT

A study was undertaken to isolate and characterize *Mycobacterium* species from black wildebeest suspected of being infected with tuberculosis in South Africa. This led to the discovery of a new *Mycobacterium avium* species, provisionally referred to as "Gnou isolate" from black wildebeest (*Connochaetus gnou*). Sixteen samples from nine black wildebeest were processed for *Mycobacterium* isolation. Following decontamination; samples were incubated in an ordinary incubator at 37°C on Löwenstein-Jensen slants and in liquid medium tubes using the BACTEC™ MGIT™ 960 system respectively. Identification of the isolate was done by standard biochemical tests and using the line probe assay from the GenoType® CM/AS kit (Hain Life Science GmbH, Nehren, Germany). The DNA extract was also analysed using gene sequencing. Partial gene sequencing and analysis of 16S rRNA gene, 16S-23S rRNA (ITS), *rpoB* and *hsp65* and phylogenetic analyses by searching GenBank using the BLAST algorithm were conducted. Phylogenetic trees were constructed using four methods, namely Bayesian inference, maximum likelihood, maximum parsimony and neighbor-joining methods. The isolate was identified as *Mycobacterium intracellulare* using the GenoType® CM/AS kit and as *Mycobacterium avium* complex (MAC) by gene sequencing. The gene sequence targeting all the genes, ITS, 16S rRNA, *rpoB* and *hsp65* and phylogenetic analyses indicated that this isolate presented a nucleotide sequence different from all currently published sequences, and its position was far enough from other MAC species to suggest that it might be a new species.

3.1 BACKGROUND

In late 2006, animals from a commercial game farm reserve in Mpumalanga Province in South Africa were harvested for game meat exportation. During meat inspection, the animal carcasses showed lesions suspicious of tuberculosis which was supported by histopathological results. The exact cause of the disease was not determined and the farm was put under quarantine for suspected bovine tuberculosis.

In February 2009, 158 animals were harvested. A high number of animals (n = 135) showed gross-visible tuberculosis-like lesions and lesions from 6 animals processed for mycobacterial cultures yielded non-tuberculous mycobacteria.

Samples (n = 16) from 9 animals were submitted to the National Health Laboratory Service (NHLS, Pretoria, South Africa) for mycobacterial isolation and a non-tuberculous mycobacterium (NTM) was isolated and identified as *Mycobacterium intracellulare* using a commercial kit and as *Mycobacterium avium* complex (MAC) by gene sequencing. The results were submitted to the Department of Agriculture, Forestry and Fisheries (DAFF, South Africa) as *M. intracellulare*, and characterization of the isolate was then done in Japan.

The complete history of the herd including the pathology report described well-developed encapsulated granulomatous lesions observed on the different samples of organs as very suspicious for bovine tuberculosis (BTB). Other lesions observed which were not typical of *M. bovis* (pseudotuberculosis) comprised lack of caseous necrosis and liquefaction in the granulomas. The inspissated material from within the capsules could almost be squeezed out in total, leaving behind an empty “shell”. There were also several smaller granulomas with a typical onion ring appearance, but absence of calcification and liquefaction with no gritty sensation on cut section of these capsules. .

The genus *Mycobacterium* contains more than 170 species (http://www.bacterio.net/mycobacterium.html), most of which are classified as NTM or potentially pathogenic mycobacteria (PPM) (Chege *et al*., 2008; Kim *et al*., 2014; Malama *et al*., 2014; Tortoli, 2014) and mycobacteria belonging to the *Mycobacterium tuberculosis* complex (MTC). MTC comprises *M. tuberculosis*, *M. bovis*, *M. africanum, M. canetti, M. pinnipedii, M. caprae, M. microti, M. mungi, Dassie bacillus, M. orygis (Oryx bacillus), M. surricatae* and the attenuated *M. bovis* Bacille-Calmette-Guerin (BCG) vaccine strain. With the exception of BCG, these species are pathogenic and can cause tuberculosis (TB) in mammalian hosts (Alexander *et al*., 2010; Helden *et al*., 2009; Pittius *et al*., 2012; Vos *et al.*, 2001).

The *M. avium-intracellulare* complex is the most commonly encountered group of NTM, and the clinically most important members are *M. intracellulare* and *M. avium* (Biet *et al*., 2005). *Mycobacterium intracellulare* has not been subdivided into subspecies whereas *M. avium* consists of four subspecies, namely *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *silvaticum* and *M. avium* subsp. *paratuberculosis*. Mycobacterium Avium Complex (MAC) includes 10 different species, namely *M. avium*, *M. intracellulare*, *M*. *colombiense*, *M. bouchedurhonense*, *M. timonense*, *M. arosiense, M. chimaera, M. vulneris, M. yongonense* and *M. marseillense* (Tortoli, 2014).

The importance of NTM has received attention during the past decade, especially in humans. NTM are found in environmental systems (such as various soil and water systems) near human settlements and can be associated with colonization, serious infection or pseudo-outbreaks with a wide variety of presentations (Biet *et al*., 2005; Kankya *et al*., 2011; Katale *et al*., 2014). Indeed in humans, the isolation of NTM from clinical samples of patients presenting with pulmonary symptoms as suspected cases of tuberculosis has increased over the years and has been observed in different countries in Africa, America and Europe (Kankya *et al*., 2011; Katale *et al*., 2014; Mirsaeidi *et al*., 2014a, 2014b; Moore *et al*., 2010) whereas in animals the clinical significance of NTM has yet to be elucidated in the disease causing process (Chege *et al*., 2008; Kankya *et al*., 2011; Katale *et al*., 2014). The members of the genus *Mycobacterium* are genetically closer to each other than the microorganisms belonging to other genera, making identification a difficult and challenging task. The management, treatment and infection control measures differ significantly between *M. tuberculosis* and NTM infections.

More than one hundred and fifty species of NTM have been reported worldwide, of which more than 60% are pathogenic to animals or humans (Kim *et al*., 2014; Tortoli, 2014). In South Africa, reports on the isolation of NTM in animals, humans and environment and their effects in disease-causing processes are limited (Gcebe *et al*., 2013; Kabongo-Kayoka *et al*., 2015; Michel *et al*., 2007; Müller *et al*., 2011).

Black wildebeest (*Connochaetus gnou*), known in Afrikaans as “Swartwildebees” and in German as “Weisschwanzgnu”, have been hunted in South Africa for meat and hides. The overall research project was mainly on tuberculosis, so samples from black wildebeest suspected of being infected with tuberculosis were processed as part of phase I of the project focusing on prevalence and molecular studies of mycobacteria. The emergence of multi-drug and extremely drug resistant *Mycobacterium tuberculosis* strains was one of the main justifications of the project. This work resulted in the reporting of a novel *Mycobacterium avium* complex species from wildebeest in South Africa, which is expected to add to the corpus of knowledge and extend the frontiers of research on NTM.

3.2 MATERIALS AND METHODS

3.2.1 Study area

Mpumalanga Province was selected as the area of study based on previous publications reporting TB in wildlife and livestock (Bengis *et al*., 1996; Michel *et al*., 2007; Vos *et al*., 2001) and availability of veterinary staff members experienced in conducting the comparative tuberculin skin test. Mpumalanga is one of the nine provinces in South Africa which is located in the north-eastern part of the country, bordering Swaziland and Mozambique to the East. It embraces the southern half of the Kruger National Park, a vast nature reserve with abundant wildlife including big game. It has a subtropical foliage supporting about 1 439 000 cattle according to the Trends in the Agricultural Sector (2013).

3.2.2 Study design and sampling

The study was designed as a cross-sectional study sampling animals in the designated area from January 2009 to January 2011. The local municipalities were selected based on the number of commercial farms, proximity to abattoirs, and location at the human-wildlife interface and the movement of animals as well. The municipalities selected comprised Malelane, Nelspruit, Lydenburg, Ermelo, Witbank and Standerton. The target population comprised cattle carcasses showing gross tuberculous-like lesions at meat inspection from positive reactors to tuberculin skin test at the municipality abattoirs during the study period. Samples from any other animal species showing gross tuberculous-like lesions were also included as convenience samples. The sampling was purposive to increase the chances of isolating mycobacteria and animals were selected based on positive reaction to the tuberculin skin test and suggestive lesions at the abattoir upon meat inspection.

3.2.3 Sources of samples

Samples from black wildebeest were received as part of phase I of a research project related to “Prevalence and molecular studies of Mycobacteria”. The samples were processed at the National Health Laboratory Service (NHLS) as part of a joint collaboration between University of Pretoria and NHLS. The history of the case was provided by the state veterinarian in charge. During a hunting period in February 2009 on a commercial game reserve located in Mpumalanga (South Africa), game animals (n=158) were randomly harvested and processed in the local abattoir according to standard operating procedures. These animals comprised black wildebeest (*Connochaetes gnou*) (n=137), blesbok (*Damaliscus dorcas phillipsi*) (n=15), blue wildebeest (*Connochaetes taurinus*) (n=2), red hartebeest (*Alcelaphus buselaphus caama*) (n=2) and springbok (*Antidorcas marsupialis*) (n=2). The animals lagging behind were the main target as well as females. During routine meat inspection of these animals, a high number of black wildebeest (n=135) showed granulomatous lesions in one or more lymph nodes or organs and this is the reason why samples were selected from this antelope species. Sixteen samples randomly selected from nine black wildebeest showing fresh lesions suggestive of tuberculosis infection were submitted to NHLS for isolation and identification of *Mycobacterium* spp. The samples included different organs and lymph nodes transported on ice (Table 3.1).

Table 3.1: Type of tissue samples from Black Wildebeest processed at NHLS

Animals	Tissue samples			
	Liver	Lung	Med	Total
ID*				
BWB 1	1	0	0	1
BWB 2	0	0	1	1
BWB 3	1	1	0	2
BWB 4	0	1	1	2
BWB 5	1	1	0	2
BWB 6	0	1	0	1
BWB 7	1	1	0	2
BWB 8	0	1	1	2
BWB 9	1	1	1	3
Tot	5	7	4	16

*ID: identity, BWB: Black Wildebeest, Med: Mediastinal lymph nodes

3.2.4 Mycobacterial isolation

Samples were frozen at -20°C until processing at NHLS. Direct impression smears were made from lesions and smears were stained using the Ziehl-Neelsen method. Tissue samples taken in a sterile manner from the inside of granulomatous lesions at the border between healthy and pathological tissues were finely cut using a sterile scalpel blade and homogenized using sterile glass beads by vortexing as described by Bengis *et al*. (1996) and Warren *et al*. (2006) with some modifications. To maximize the mycobacterial yield, specimens were subjected to a digestion and decontamination procedure using N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) solution with NaOH at a final concentration of 2% (Chatterjee *et al*., 2013). The specimens were left at room temperature for 15 min during the decontamination process and thereafter neutralized with phosphate buffer, centrifuged (Beckman Coulter) at 3000 x g for 15 min at 4°C, and the supernatant decanted and pellet suspended into 1 mL of phosphate buffer. The sediment was inoculated onto two LJ slants supplemented with pyruvate and glycerol and an antibiotic mixture of polymyxin B, amphotericin B, carbenicillin and Trimethoprim (PACT) (National Health Laboratory Service, South Africa, and Becton Dickinson, Germany) using a 0.01 mL calibrated loop. A further 0.5 mL of the sediment was inoculated with a graduated

Pasteur pipette into a prepared liquid medium tube (BBL™ MGIT™ Mycobacterium Growth indicator tubes) enriched with OADC and containing 800 µL of PANTA™ antibiotic mixture (BD™). This was incubated in the BACTEC™ MGIT™ 960 mycobacterial detection system at 37°C (Warren *et al*., 2006). The system was monitored for a maximum period of 42 days for bacterial growth whereas LJ slants were observed for colony growth and any other contaminant at two week intervals for 10 weeks. Tubes detected as positive within that period were further processed using Ziehl-Neelsen staining and examined microscopically for the presence of acid fast organisms and morphology. Thereafter, they were subcultured on LJ slant supplemented with glycerol and pyruvate. For identification purposes, a single colony was subcultured on a fresh LJ slant to obtain pure colonies. The same was repeated with two other colonies on different LJ slants to rule out the possibility of missing a different organism. Reference cultures of *M. avium* (ATCC 25291), *M. bovis* BCG and *M. tuberculosis* (ATCC 25177) were used as positive controls.

3.2.5 Mycobacterial identification

3.2.5.1 Biochemical profile

Colonies were suspended in Middlebrook 7H9 (M7H9) broth enriched with OADC and inoculated into the different biochemical substrates according to the manufacturer's instructions (Mycobacterium identification kit, Kyokuto Pharmaceutical Industrial Co., Ltd., Japan).

3.2.5.2 DNA extract and primary molecular identification

DNA extracts were prepared based on the GenoType CM/AS reverse line blot assay (Hain Life Science, Gmbh Nehren, Germany). DNA was extracted from colonies on LJ slants by heating at 95°C for 20 min in a water bath. Primers provided by the manufacturer's kit (Hain Life Science, Gmbh Nehren, Germany) were used. The formula for the PCR assay per tube mixture contained 1.1 µL of ultra-pure water, 5 µL of 10 x buffer, 3.6 µL of $MgCl_2$ (25 mM), 35 µL of primer/nucleotide mix from the kit and 0.3 µL of hot Taq polymerase. The thermocycler was programmed to initiate the PCR as follows: one cycle of 15 min at 95°C, followed by 10 cycles of 30 sec at 95°C, 2 min at 58°C, 25 sec at 95°C; then 20 cycles of 40 sec at 53°C and 40 sec at 70°C with a final cycle of 8 min at 70°C. The hybridization was then followed as per manufacturer's instructions using the strips provided in the kit (DNA strip

Mycobacterium identification species). Part of each DNA extract was also stored at -20°C prior to further investigations in Japan.

3.2.6 Gene sequencing

The DNA extract obtained during the primary identification was processed further by sequencing analysis; targeting *hsp65*, *rpoB*, 16S rRNA genes and 16S-23S rRNA internal transcribed spacer (ITS) for identification of the bacterial species.

3.2.6.1 16S ribosomal RNA gene and ITS

The 16S ribosomal RNA gene and flanking 16S–23S rRNA ITS region was amplified with a primer pair of Bact-rrs-F: 5'-AGAGTTTGATCCTGGCTCAG- 3' and myco ITS-23S-Rv: 5'-CGGTTGACAGCTCCCCGAGGC- 3'. Amplification reaction mixture consisted of 0.5 μM of each primer pair, 1× ExTaq buffer (Takara Bio Inc., Japan), 0.5 M betaine, 0.25 mM each of dNTPs, 1 U of ExTaq DNA polymerase (Takara Bio Inc.), 1 μL of target DNA solution and milli-Q water to adjust the final volume to 20 μL. Amplification was performed in a thermal cycler with a pre-heat step at 98°C for 1 min, 38 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 10 sec and extension at 72°C for 110 sec followed by a final extension at 72°C for 5 min. The amplicon was electrophoresed on a 1% agarose gel and a band of approximately 1.9 Kbp was excised and purified with Wizard SV Gel and PCR Clean-Up System (Promega Corp., USA). The sequence of the 5' region of the 16S ribosomal RNA gene and ITS were read with primers Bact-rrs-F and myco ITS-23S-Rv, respectively, by ABI PRISM 3130*xl* Genetic Analyzer (Life Technologies Corp., CA, U.S.A.) according to the manufacturer's instructions.

3.2.6.2 rpoB

Partial *rpoB* gene was amplified and sequenced with primers Myco-F and Myco-R designed by Ben Salah *et al*. (2008) PCR reaction mixture was comprised of 0.5 μM of each primers, 1× GoTaq buffer (Promega Corp., USA), 0.5 M betaine, 0.25 mM each of dNTPs, 1 U of GoTaq DNA polymerase (Promega Corp.), 1 μL of target DNA solution and milli-Q water to adjust the final volume to 20 μL. Amplification was performed with an initial denaturation at 96°C for 1 min, 38 cycles of denaturation at 96°C for 10 sec, annealing at 60°C for 10 sec and extension at 72°C for 45 seconds followed by a final extension at 72°C for 5 min. The amplicon was electrophoresed on a 1% agarose gel and a band with a size of 761 bp was excised and purified. The sequence was read by ABI PRISM 3130*xl* Genetic Analyzer (Life Technologies Corp.) according to the manufacturer's instructions.

3.2.6.3 hsp65

Partial *hsp65* gene was amplified and sequenced with primers Myco-hsp65F (565-585): 5'- AGGGTATGCGGTTCGACAAG- 3' and MAC hsp65R (Turenne *et al*., 2006). Amplification was performed with an initial denaturation at 96°C for 1 min, 38 cycles of denaturation at 96°C for 10 sec, annealing at 53°C for 10 sec and extension at 72°C for 45 sec followed by a final extension at 72°C for 5 min in the same reaction mixture content described in section 3.2.6.2. The band with a size of 1067 bp was excised and sequencing was performed by the same procedure as in section 3.2.6.2.

The obtained sequences were compared with the GenBank nucleotide database by the blastn program (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov).

3.2.7 Phylogenetic analyses

The datasets for the different genes (ITS, 16S, *hsp65* and *rpoB*) were collated by searching GenBank (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm, an acronym for the Basic Local Alignment Search Tool (Altschul *et al*., 1990). BLAST searches various databases, as specified by the user, to look for similar sequences, and then uses a similarity matrix to measure the similarity between sequences and the possibility that the similarity could be due to chance based on the nucleotide sequence of the query versus its target. There are several types of searches available but for these analyses "blastn" was used which searches nucleotide databases with a nucleotide query. These sequences were then downloaded into a local database using BioEdit version 7.2.1 (Hall, 1999). Sequences were aligned using MAFFT version 7 (Katoh *et al*., 2005, 2002) http://www.mafft.cbrc.jp/alignment/server/) with default parameters. Multiple Alignments by Fast Fourier Transformation (MAFFT) utilizes an iterative algorithm, unlike previous progressive alignment methods. Where necessary, small adjustments were made to the alignments manually to minimize hypothesized insertion/deletion events, again using BioEdit software (Hall, 1999). The programme jModelTest 2.1.6 (Darriba *et al*., 2012) was run for each gene separately, as well as the concatenated dataset, to determine the best evolutionary model to use for each dataset. Phylogenetic trees were constructed for all data sets by four different methods, Bayesian inference (BI), maximum likelihood (ML), maximum parsimony (MP) and neighbor-joining (NJ). This not only allowed independent confirmation of results, but also the benefit of different methods with different strengths, weaknesses and sensitivities. NJ and MP analyses were conducted with MEGA 5 (Molecular Evolutionary Genetic Analysis) (Tamura *et al*., 2011). PhyML (Guindon *et al*., 2010) was used to carry out ML analyses on all the datasets and Mr. Bayes v3.2.5 (Ronquist *et al*., 2012) was used on the concatenated dataset. Both NJ and ML analyses used the relevant evolutionary model as given in jModelTest. All analyses, except those by Bayesian inference, consisted of 1000 bootstrap replications (Felsenstein, 1985), a statistical method for testing the reliability of all the groupings within the various trees.

3.2.8 Statistical analyses

The agreement between databases was calculated using Cohen's Kappa. To compare the results obtained from each gene and combination of two genes, only the databases identified with better performance in the first analysis were used. One thousand bootstrap replications were used for testing the reliability of all the groupings within the various trees (Hallgren, 2012; Joao *et al*., 2014).

3.2.9 GenBank accession numbers

The sequences generated in this study were deposited in the GenBank database under accession number KR856202 for *hsp65*, KR856203 for *rpoB*, KR856204 for 16S rRNA and KR856205 for 16S-23S rRNA ITS.

3.3 RESULTS

3.3.1 Mycobacterial isolation

A mediastinal lymph node (from 1 animal) out of 16 samples (from 9 animals) yielded similar smooth colonies of slow growing microorganisms on LJ slants inoculated with the sediment from positive MGIT tubes. The acid-fast rods were observed under the microscope.

3.3.2 Biochemical characteristics

The phenotypic characteristics, including growth rate (fast/slow), production of pigment, growth at different temperatures and biochemical reactions with relevant substrates were evaluated. Colonies were identified as MAC slow growers with no pigment on specific substrates. They were positive for tellurite reduction, stimulation by pyruvate and heat-stable catalase but negative for niacin production, nitrate reduction, urease, acid phosphatase activity, Tween 80 hydrolysis (7 and 14 days) and urease (Table 3.2); (Cook *et al*., 2003).

Table 3.2: Growth and biochemical characteristics of the isolate

Growth rate	
27°C	Positive
37°C	Positive (Slow growth)
45°C	Negative
Stimulation by pyruvate	Positive
Pigmentation	No pigmentation
Niacin production	Negative
Nitrate reduction	Negative
Catalase production	Positive
Tellurite reduction	Positive
Urease	Negative
Acid phosphatase activity	Negative
Tween 80 hydrolysis (7 and 14 days)	Negative

3.3.3 Primary molecular identification

The isolate was identified as *Mycobacterium intracellulare* using a commercial kit routinely used at NHLS, the GenoType Mycobacterium CM/AS (Hain Life Science, Gmbh Nehren, Germany).

3.3.4 Phylogenetic analyses

3.3.4.1 ITS sequences

Alignment of the 16S-23S rRNA ITS of *Mycobacterium* “Gnou isolate” with the other sequevars of the MAC show that the Gnou isolate is most closely related to the MAC-T sequevar with only three nucleotide differences.

Phylogenetic analyses of these data by NJ, MP and ML, placed the Gnou isolate within the MAC and more closely related to MAC-T, with MAC-M, MAC-I and MAC-L forming a sister group. All three methods placed the Gnou isolate in the same place with varying degrees of bootstrap support.

3.3.4.2 16S rRNA, hsp65 and rpoB analyses.

All three datasets by every method grouped the Gnou isolate with *M. vulneris, M. bouchedurhonense,* and *M. colombiense* with *M. arosiense* included in the group by just the MP analysis of *hsp65*. However, all other analyses placed *M. arosiense* close to the Gnou isolate grouping. With the similarity between the various trees the four genes were combined into one dataset to give a concatenated tree (Figure 3.1). Generally the groupings all have good bootstrap support. All the species above *M. mantenii* in this phylogeny are members of the MAC.

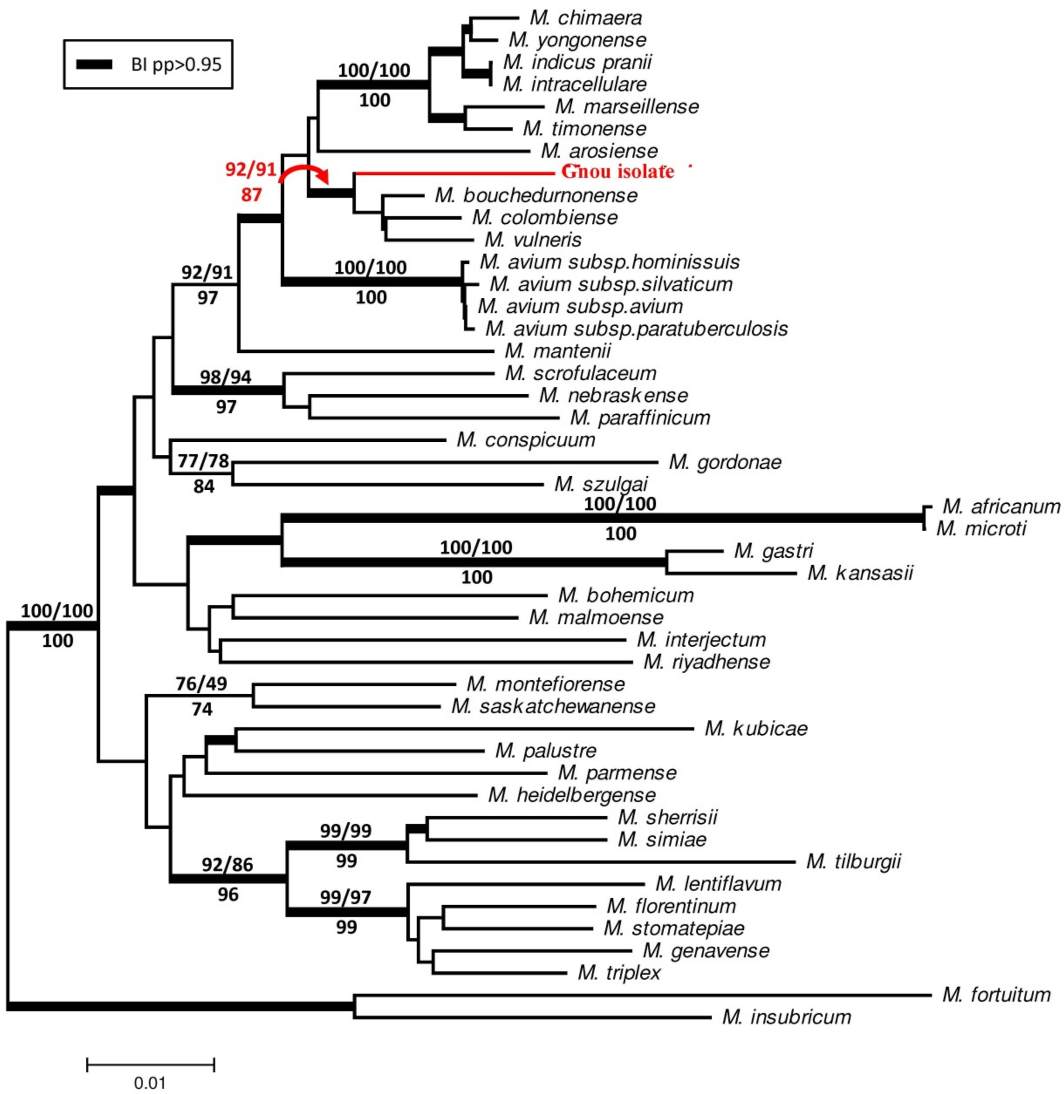


Figure 3.1: Phylogenetic tree obtained by NJ analysis of concatenated nucleotide sequences of 16S rRNA, 16S-23S rRNA, *hsp65* and *rpoB*. Bootstrap values (1000 replicates) are given above the branches for NJ/ML and below for MP. Branch support values on the nodes are shown in percentage and the values less than 60% are not shown. The tree is rooted with *M. fortuitum* and *M. insubricum*.

3.4 DISCUSSION AND CONCLUSION

This is the first report in South Africa of the discovery of a novel *Mycobacterium avium* complex species from black wildebeest which has been named "Gnou isolate" to reflect the species name of the wildebeest from which it was isolated. Our isolate was identified using analysis of 16S rRNA gene, *hsp65*, *rpoB* and ITS. A number of studies suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65 to 83%) (Janda and

Abbott, 2007; Joao *et al*., 2014). Although 16S rRNA gene sequencing is highly useful in regards to bacterial classification, it has low phylogenetic power at the species level and poor discriminatory power for some genera (Janda and Abbott, 2007). It has also been reported that analysis of 16S rRNA gene alone is insufficient for the accurate identification of NTM (Joao *et al*., 2014); it was thus proposed that a stepwise algorithm combining 16S rRNA and *hsp65* gene analysis by multiple public databases could be used to identify NTM at the species level. In some cases, 16S rRNA gene sequence data cannot provide a definitive answer since it cannot distinguish between recently diverged species. While it is impossible to be completely accurate when modelling all evolution that has occurred between a set of sequences, several parameters appear to be particularly important. These are corrections for substitution patterns (nucleotide substitution matrices) and correction for different evolutionary rates at different sites, most accurately corrected using a gamma distribution model, the shape parameter α of which is calculated by jModelTest. It has been established that NTM cause disease (Kim *et al*., 2014), but the significance of NTM in the disease processes in animals should be investigated further as the presence of a small number of a specific pathogen does not correlate with the virulence of the pathogen nor its economic importance. Organisms act in synergy, potentiating the colonization by other bacteria. Although mixed infection with NTM has been reported, attention should be given to NTM in the future as several studies have recognized the significance of NTM as a major public health issue around the world (Kankya *et al*., 2011; Kim *et al*., 2014; Malama *et al*., 2014; Moore *et al*., 2010; Temmerman *et al*., 2014). Furthermore, NTM have been found to interfere with the diagnosis of TB in cattle; indeed, some cross reactions between the antigens of NTM with those used for diagnostic purposes such as *M. avium* and *M. fortuitum* may compromise the diagnosis and control of bovine tuberculosis (De la Rua-Domenech *et al*., 2006; Gcebe *et al*., 2013).

To our knowledge, this is the first report of this new isolate of NTM, “Gnou isolate”, sampled from black wildebeest (*Connochaetes gnou*) in South Africa. The diagnosis of NTM should be standardized as the isolate was identified as *Mycobacterium intracellulare* using DNA strip according to Hain’s method at National Health Laboratory Service and confirmed as a new species of MAC by gene sequencing at Research Center for Zoonosis Control at Hokkaido University in Japan. Tortoli *et al*.

(2010) reported the misidentification of NTM using commercial kit assays as we have also experienced with this study. The partial sequencing of 16S rRNA gene alone is not sufficient to fully identify NTM to species level; algorithm analysis combining all four genes should be considered as well as biochemical identification. One of the characteristics of this isolate was slow growth. The role of NTM, in particular slow growing MAC, should be further investigated in the disease-causing process as they could potentiate colonization by other rapidly growing microorganisms. The isolation and identification at species level of some NTM should be performed to establish their clinical relevance in animals and humans. Countries should also be encouraged to register new species on the international data bank for purposes of adding to the epidemiological knowledge of this genus.

Postscript

The next chapter comprises the start of phase 2 of the project whereby extracts of plant species from the family Anacardiaceae were tested for antimycobacterial activity against a panel of *Mycobacterium* species, both rapidly growing saprophytic and slow-growing pathogenic species. Cytotoxicity of the extracts was also tested against various cell lines. The chapter has been published in the Journal Phytotherapy Research. The article is attached in Appendix C.

CHAPTER 4:
ANTIMYCOBACTERIAL ACTIVITY AND CYTOTOXICITY OF LEAF EXTRACTS OF SOME AFRICAN ANACARDIACEAE TREE SPECIES

ABSTRACT

Treatment of tuberculosis (TB) is a challenge due to multi- and extremely drug resistant strains of *Mycobacterium tuberculosis*. Plant species contain antimicrobial compounds that may lead to new anti-TB drugs. Previous screening of some tree species from the Anacardiaceae family revealed the presence of antimicrobial activity, justifying further investigations. Leaf extracts of 15 Anacardiaceae tree species were screened for antimycobacterial activity using a twofold serial microdilution assay against the pathogenic *Mycobacterium bovis* and multidrug resistant *M. tuberculosis* and rapidly growing mycobacteria, *M. smegmatis, M. fortuitum* and *M. aurum.* The vaccine strain, *M. bovis* and an avirulent strain, *M. tuberculosis* H37Ra, were also used. Cytotoxicity was assessed using a colorimetric assay against Vero kidney, human hepatoma and murine macrophage cells. Four out of 15 crude acetone extracts showed significant antimycobacterial activity with minimum inhibitory concentration (MIC) values varying from 50 to100 µg/mL. *Searsia undulata* had the highest activity against most mycobacteria, followed by *Protorhus longifolia*. *Mycobacterium fortuitum* was the strongest predictor of activity against MDR-TB (correlation coefficient = 0.65). Bioautography against *M. aurum* and *M. fortuitum* worked well as indicators of the R_f values of active compounds yielding strong zones of inhibition. The leaf extracts of *S. undulata* and *P. longifolia* each had more than ten different antimycobacterial compounds and had low cytotoxicity with LC_{50} values above 100 µg/mL.

4.1 INTRODUCTION

Tuberculosis occurs in humans, cattle, wild animals and many other domesticated species. The *Mycobacterium tuberculosis* complex (MTC) comprises *M. tuberculosis, M. bovis, M. africanum, M. canetti, M. pinnipedii, M. caprae, M. microti, M. mungi, Dassie bacillus, M. orygis (Oryx bacillus), M. surricatae* and the attenuated *M. bovis* Bacille-Calmette-Guerin (BCG) vaccine strain. With the exception of BCG, these species are pathogenic and can cause tuberculosis (TB) in mammalian hosts

(Alexander *et al*., 2010; Kabongo-Kayoka *et al*., 2015; Pittius *et al*., 2012; Vos *et al*., 2001).

Despite the fact that TB can be cured, this disease remains a worldwide public health threat due to the emergence of multi- and extremely drug resistant strains and subsequent poor response to current antibiotics. One third of the world's population is infected with *Mycobacterium tuberculosis* (MTB) and is hence at risk of developing active TB (WHO, 2015). In the order of 9.6 million people every year develop TB and 1.5 million die from the disease. This includes 0.4 million TB deaths among HIV positive people (WHO, 2015). From the 9.6 million, only 12% were HIV positive and it was also estimated that 480 000 cases of multidrug-resistant TB (MDR-TB) have occurred in 2014 (WHO, 2015). This confirms that TB alone is still a major challenge.

There is currently no effective vaccine to protect against TB. In humans, control of the disease relies heavily on detecting infectious cases and treating them for at least six months with a combination of antibiotics. The course of antibiotics involves the administration of first-line anti-TB drugs, which are combinations of isoniazid (INH), rifampicin (RIF), pyrazinamide and ethambutol for 2 months to kill the rapidly growing bacteria. The treatment is continued in the next 4 months with a combination of INH and RIF because of their sterilizing activity to eliminate bacilli which are dormant in the macrophages, or slow growers (Rivers and Mancera, 2008). Toxicity associated with these first-line drugs and the long duration of treatment has led to low patient compliance, giving rise to drug-resistant strains. Multidrug-resistant mycobacteria are resistant to INH and RIF whereas extensively drug-resistant mycobacteria are resistant to second-line drugs such as fluoroquinolones and to at least one injectable drug in addition to INH and RIF. Hence, there is a need to find new anti-tubercular agents with novel modes of actions (Chinsembu, 2016).

It is most likely that the emergence of resistant *Mycobacterium* isolates will continue in the future, exhausting the current range of effective antibiotics available. Therefore, new classes of anti-TB agents are urgently needed, and research projects seeking for alternative therapeutics have been encouraged. Natural resources have been explored to develop new antibiotics. Substances that can either inhibit the growth of pathogens or kill them while having little or no toxicity to host cells are considered good candidates for developing new antimicrobial drugs (Nguta *et al*., 2016).

Medicinal plants are used in many parts of the world to treat different ailments including TB-related symptoms such as chest complaints, respiratory ailments, fever and coughing (McGaw *et al*., 2008a, 2008b). Relevant reviews of traditional medicinal use of plants in Africa are available (Hutchings *et al*., 1996; Kuete, 2010; Maroyi, 2013; Nguta *et al*., 2016; Stark *et al*., 2013; Van Wyk *et al*., 1997).

South Africa possesses the richest temperate flora in the world, with an estimated 24 000 species and intraspecific taxa in 368 families more than 10% of the world's vascular plant flora (Germishuizen and Meyer, 2003). There is much potential for discovery of structurally interesting metabolites with activity against *Mycobacterium* species from these plants. The existence of a strong cultural reliance on traditional medicines within South African communities justifies the importance of studying their safety and efficacy (Fennell *et al*., 2004).

In a study conducted on Indian medicinal plants, Gautam *et al*. (2007) reported that with many of the plant species examined, there was a strong positive correlation between antimycobacterial activity and ethnomedicinal use for TB and TB-related diseases. Although this provides support for investigating plants customarily used in other cultures to treat symptoms relating to TB, other plants tested for antimycobacterial activity using indicator organisms such as *M. smegmatis* and *M. bovis* BCG should also be investigated (McGaw *et al*., 2008a). Testing plant material not only against ATCC strains but against current isolates is crucial as these microorganisms are part of the changing environment harbouring emerging resistant strains.

The Anacardiaceae, cashew or Sumac is a tropical family that comprises flowering plant bearing fruits that are drupes and in some cases producing urushiol which is an irritant substance (Mabberley, 1997). The Anacardiaceae comprises several genera and species of economic importance as shown in Figure 4.1 (a-l) namely Cashew (*Anacardium occidentale*), Mango (*Mangifera indica*), Poison Ivy (*Toxicodendron radican, Smodingium argutum*), Sumac (*Searsia* species), Smoke tree (*Cotinus coggygria, Cotinus obovatus*), Marula (*Sclerocarya birrea*), Yellow mombin

(*Spondias mombin, Spondias purpurea* var. *lutea*), Cuachalalate (*Amphipterygium adstringens*) and Pistacia (*Pistacia lentiscus, Pistacia integerrima*). This family, which belongs to the order Sapindales, is represented by 77 genera and 600 species mostly distributed in tropical, subtropical, and temperate areas (Mabberley, 1997). Very few plant species of the Anacardiaceae family have been investigated for antimycobacterial activity. One of the objectives of this study was to investigate *in vitro* antimycobacterial activities as well as cytotoxicity of selected plant species from the Anacardiaceae family. These plants were selected mainly based on a preliminary broad antimicrobial screening (Pauw, 2014).

A summary on ethnobotanical use of the selected plant species from the Anacardiaceae family is presented in Table 4.1. Eight out of the 15 plants, namely *Lannea discolor, Loxostylis alata, Ozoroa mucronata, Ozoroa paniculosa, Protorhus longifolia, Searsia chirindensis, Searsia lancea* and *Sclerocarya birrea*, have antimicrobial properties mainly against Gram-negative and Gram-positive organisms (Ahmed *et al*., 2014; Hutchings *et al*., 1996; Mosa *et al*., 2014; Stark *et al*., 2013; Van Wyk *et al*., 1997). Three species (*Searsia chirindensis, Searsia undulata* and *Smodingium argutum*) have good anti-inflammatory activity (Hutchings *et al*., 1996; Ojewole, 2008; Stark *et al*., 2013). It would be useful to identify plant species with both antimycobacterial and anti-inflammatory efficacy as both acute and chronic inflammation are induced during the course of tuberculosis disease progression (Volpe *et al*., 2006).

4.2 MATERIALS AND METHODS

4.2.1 Source of plant materials and extraction

Leaves of the 15 plant species were collected in November 2009 from the Lowveld National Botanical Gardens in Nelspruit, the University of Pretoria Botanical Gardens and the Pretoria National Botanical Garden, South Africa in terms of a signed material transfer agreement. Leaves were kept in labelled bags with full identification and collection dates. Voucher specimens were kept in the Lowveld National Botanical Gardens (Nelspruit), HGWJ Schweickerdt Herbarium of the University of Pretoria and Phytomedicine Programme, University of Pretoria (Table 4.1). The leaves were air-dried at room temperature suspended in bags of net material in a ventilated room (refers Figure 4.1a), ground to a fine powder in a

Macsalab Mill (Model 200 LAB Eriez®, Bramley, Johannesburg, South Africa) and stored in closed glass containers away from light until needed. Three grams of each plant were extracted in 30 mL of acetone (technical grade, Merck, Darmstadt, Germany) in a 50 mL polypropylene centrifuge tube. Acetone was used as the extracting solvent due to its ability to extract a wide range of compounds and low toxicity on different microorganisms (Eloff, 1998a). The tube was vigorously shaken for 30 min on an orbital shaker, then centrifuged at 4000 × g for 10 min and the supernatant was filtered using Whatman No.1, 110mm diameter filter paper, before being transferred into pre-weighed glass containers as shown in Figure 4.1 (b) (Eloff, 1998a). The extraction was repeated thrice on the same plant material and the solvent was removed by evaporation under a stream of air in a fume hood at room temperature to produce the dried extract. Extracts were made up to a concentration of 10 mg/mL in acetone for use in relevant tests.

(a) **(b)**

Figure 4.1: Air drying of leaves suspended in bags (a), filtration of the acetone extracts for preliminary assays (b)

Table 4.1: Ethnomedicinal use and known isolated compounds of selected species of the Anacardiaceae family

Botanical names	Common names	Voucher number	Plant part used	Ethnomedicinal use	Phytochemical constituents /pharmacological activities	References
Harpephyllum caffrum Bernh.	Wild plum (Eng); wildepruim (Afr.); Umgwenya (X, Z)	PRU2583	Bark, leaves and roots	Purifying blood, skin problems scarifications around sprains and fractures and leg paralysis due to poison	Polyphenolic and flavonoids/anticonvulsant	Hutchings *et al*., 1996; Van Wyk *et al*., 1997
Heeria argentea Meisn.	Cape Rockwood, Wild Apricot (Eng.); Kliphout (gom) and Wilde-Naelboom (Afr.)	PMDN751	Gum	Gum mixed with sweet oil used as plaster for burns, wounds, tender nipples and powdered herb with fat applied to ulcers	__	Hutchings *et al*., 1996
Lannea discolor (Sond.) Engl.	Live long (Eng); Dikbas (Afr.); Isiganganyane (Z)	PMDN565	Bark, roots and leaves	Treatment of diarrhoea, abscesses, infertility in women, menorrhagia, gonorrhea, swollen legs, whooping cough, counteract effect of lightning and sore eyes	Phenolic flavone/antimicrobial	Hutchings *et al*., 1996; Maroyi, 2013; Van Wyk *et al*., 1997

Loxostylis alata A. Spreng. ex Rchb.	Wild pepper tree, tarwood (Eng); teerhout, tierhout (Afr); Ufutho, ufuthu (Z)	PRU3526	Bark and leaves	Used at childbirth boost immunity	Phenolic lipids, cardanol and ginkgolic acid; lupeol 1 and B-sitosterol 2; antimicrobials	Ahmed *et al*., 2014
Ozoroa mucronata (Bernh.) R. Fern. & A. Fern.	No common names associated with this taxon yet. Mvunda-Jukunu in Tanzania	NEL71/1993	Leaves and roots	Diarrhoea, dysentery, stomach troubles, gonorrhea, bilharzia and antidote for snake bite	Anacardic acid; flavonoids, phenols, tannins/antimicrobial	Ahmed *et al*., 2014
Ozoroa paniculosa (Sond.) R. Fern. & A. Fern.	Red resin tree (Eng.)	PMDN568	Bark and roots	Diarrhoea and abdominal pain	Flavonoids, phenols, tannins Anacardic acid, ginkgoic acid and triterpenes/antimicrobial	Ahmed *et al*., 2014; Stark *et al*., 2013
Protorhus longifolia (Bernh.) Engl.	Red beech (Eng), rooiboekenhout (Afr.); umkhomizo, unhlangothi (Z)	PRU120030	Leaves, stem bark and seeds	Heartburn, bleeding from stomach, depilatories, Heartwater, dysentery and diarrhoea in cattle	Triterpenes, lanosteryl triterpen/antimicrobial, Antihyperlipidemic and antihyperglycemic	Mosa *et al*., 2014
Searsia chirindensis (Baker f.) Moffett	Red currant (Eng.); Bostaaibos (Afr.), umHlabamvubu (Z)	PMDN333	Stem Bark	Heart, stomach ailments, diarrhoea, strengthen the body, stimulate circulation, rheumatism and mental disturbances	Saponins, tannins triterpenoids and flavonoids/ Inflammatory, analgesic and anticonvulsant	Ojewole, 2008
Searsia lancea (L.f.) F.A.Barkley	Karee (Eng.), umHlakotshane (X)	PMDN750	Bark and roots	Diarrhoea, gallsickness	Flavonoids and tannins/antimicrobial	Stark *et al*., 2013

Searsia leptodictya (Diels.) T.S.Yi., A.J.Mill.& J.Wen	Mountain karee (Eng.), bergkaree (Afr.) Motshotlwane (Tsw)	NEL94/1969	Bark, leaves and roots	Diarrhoea and pain	Phenols, tannins and flavonoids	Ahmed *et al*., 2014; Stark *et al*., 2013
Searsia magaliesmontana (Sond.) Moffett		NEL68/1997	—	—	—	—
Searsia pyroides (Burch.) Moffett	Wildcurrant (Eng.); Brandtaaibos, (Afr.); Inhlokoshiyane (Z)	PMDN570	Leaves, roots and stem bark	Treatment of epilepsy and cough medicine	Flavonoids bichalcones	Maroyi, 2013
Searsia undulata (Jacq). T.S.Yi, A.J.Mill. & J.Wen	T'kuni (khoi); Kuni bush, (Eng.); Koenibos (Afr.)	PRU120031	Bark, leaves and roots	Post-parturient problems and treatment for chest colds	Apigenin 7,4´-dimethyl ether, flavone/ Anti-inflammatory	Fourie and Snyckers, 1984; Hutchings et al., 1996)
Sclerocarya birrea subsp. *caffra* (Sond.) Kokwaro	Cider tree, marula (Eng.); Maroela (Afr.); Umganu (Z)	NEL103/1967	Bark, leaves and roots	Diarrhoea, dysentery, pain, stomach problems, fever, cough, malaria, tonic, diabetes and sore eyes	Gallotannins, Flavonoids, epicatechin, alkaloids, triterpenoids and vitamin c /Anticonvulsants; antidiarrhoeal, antidiabetic, anti-inflammatory, antimicrobial and antioxidant	Maroyi, 2013; Stark *et al*., 2013; Van Wyk *et al*., 1997
Smodingium argutum E. Mey. ex Sond.	African poison ivy (Eng.); Pynbos (Afr.); Umthomvane (Z)	PMDN188	Bark, leaves and roots	Diarrhoea and diabetes	Catechols, phenolic lipids (cardanol and ginkgolic acid) /Antidiarrhoeal, hypoglycaemic effects and anti-inflammatory	Hutchings *et al*., 1996

(a) (b)

Figure 4.2: Leaves, flowers and fruits of *Anacardiacea occidentalis* (Cashew tree) (a) and *Lannea discolor* (b)

Source: courtesy of www.zipcodezoo.com and www.tropical.theferns

(c) (d)

Figure 4.3: Leaves and fruits of *Mangifera indica* (c) and *Ozoroa paniculosa* (d)

Source: courtesy of www.alamy.com

(e)

(f)

Figure 4.4: Leaves of *Ozoroa mucronata* (e) and leaves and fruits of *Heeria argentea* (f)

Source: courtesy of www.ispotnature.org

(g)

(h)

Figure 4.5: Leaves and fruits of *Searsia chirendensis* (g) and leaves of *Searsia lancea* (h)

Source: courtesy of www.plantzafrica.com and www.redlist.sanbi.org

(i) (j)

Figure 4.6: Leaves of *Searsia magaliesmontana* (i) and *Smodingium argutum* (j).

Source: courtesy of www.operationwildflower.org.za

(k) (l)

Figure 4.7: Leaves and flowers of *Searsia pyroides* (k) and *Protorhus longifolia* (l).

Source: courtesy of www.bidorbuy.co.za

4.2.2 Antimycobacterial activity

The antimycobacterial activity of the acetone crude extracts of leaf powder was determined using bioautography and the two-fold serial microdilution method. The pathogenic and rapidly growing mycobacteria were test microorganisms used for antimycobacterial activity.

4.2.2.1 Pathogenic mycobacteria

4.2.2.1.1 Mycobacterium tuberculosis multi-drug resistant isolates (MDR-TB)

Two clinical isolates of multi-drug resistant *Mycobacterium tuberculosis* (MDR-TB) were used. Both isolates were obtained from patients admitted to the MDR-TB ward at Tshepong Hospital in Klerksdorp, North West Province of South Africa in December 2012. The patients were a 34 year old female and an 18 year old male. Samples of sputum had been submitted to the National Health Laboratory Service (NHLS) in Pretoria for culture in liquid medium and PCR/Line Probe Assay. Both isolates were resistant to isoniazid and rifampicin.

4.2.2.1.2 Mycobacterium bovis

Mycobacterium bovis cultures were isolated from field samples of cattle from herds that tested positive on tuberculination skin test during the period January 2009 to January 2011(Kabongo-Kayoka *et al*., 2015) as described in Chapter 2 under section 2.3.2.

4.2.2.2 Rapidly growing mycobacteria

Mycobacterium bovis BCG (Pasteur strain P1172) was obtained from the Tuberculosis Laboratory at the ARC-Onderstepoort Veterinary Institute and *M. tuberculosis* H37Ra, which is routinely used as reference material at NHLS, was obtained from the American Type Culture Collection (ATCC) number 25177. Other reference strains used included a *M. aurum* strain from the National Collection of Type Cultures (NCTC), a culture collection of Public Health England, *M. aurum* (NCTC 10437), *M. smegmatis* (ATCC 1441) and *M. fortuitum* (ATCC 6841).

4.2.2.3 Maintenance of cultures

Fresh cultures were used in the relevant assays. The pathogenic isolates of *Mycobacterium* spp. were kept at room temperature on LJ slants supplemented with glycerol, or pyruvate in the case of the *M. bovis* BCG cultures, and were used within a month. Prior to each assay, cultures were reactivated in liquid medium, Middlebrook 7H9, using MGIT 960 tubes which were incubated at 37°C in the BACTEC MGIT 960 instrument, in which they were automatically monitored each hour for fluorescence development for 42 days or until a positive signal developed. Bacterial suspensions from MGIT tubes were then subcultured on solid medium LJ slants with pyruvate for *M. bovis* and LJ slants with glycerol for *M. tuberculosis*. LJ tubes were then incubated in a walk-in incubator at 37°C for 4 to 6 weeks. A stained Ziehl-Neelsen smear was made from the sediment of the MGIT tube and the slant of LJ medium. Reference cultures of *M. avium* (ATCC 25291), *M. bovis* BCG and *M. tuberculosis* H37Ra (ATCC 25177) were used as positive controls.

The rapidly growing *Mycobacterium* species were maintained on Middlebrook 7H10 agar prepared according to the manufacturer instructions (Merck) and kept in a fridge at 4°C until used within a month. *Mycobacterium aurum* and *M. fortuitum* were revived using Middlebrook 7H9 broth with glycerol and tween and supplemented with OADC (Oleic acid Albumin Dextrose Catalase) whereas *M. smegmatis* was revived using MH broth (Merck). Bacterial suspensions were inoculated on MH agar plates for *M. smegmatis* and the rest on Middlebrook 7H9 agar. All agar plates were then incubated at 37°C until colony growth was visible within 24 to 72 hours depending on the growth rate of the specific mycobacterial species. The final density of 1 x 10^5 CFU/mL used was confirmed after spreading an inoculum of 100 µL of tenfold serial dilutions of the bacterial suspensions at McFarland No 1 onto Middlebrook 7H10 (M7H10) agar plates incubated at 37°C and counting colonies.

4.2.2.4 Minimum inhibitory concentration (MIC) determination

The MIC values were determined using the serial microplate method developed by Eloff (1998b) and slightly modified for mycobacteria by McGaw *et al*. (2008b). The MIC value was the lowest concentration of the tested extract or sample inhibiting the growth of the *Mycobacterium* species under investigation.

Mycobacterial suspensions were prepared from a pure culture of fresh colonies from solid medium and suspended in Middlebrook 7H9 (M7H9) liquid medium supplemented with 10% OADC. These colonies were transferred into a sterile screw-capped tube containing 3 mL of M7H9 broth and homogenized by placing the tube on a Vortex mixer for 5 minutes. After the larger particles had settled, the mycobacterial suspension was adjusted to McFarland no.1 turbidity standard by adding more broth (Lall and Meyer, 1999).

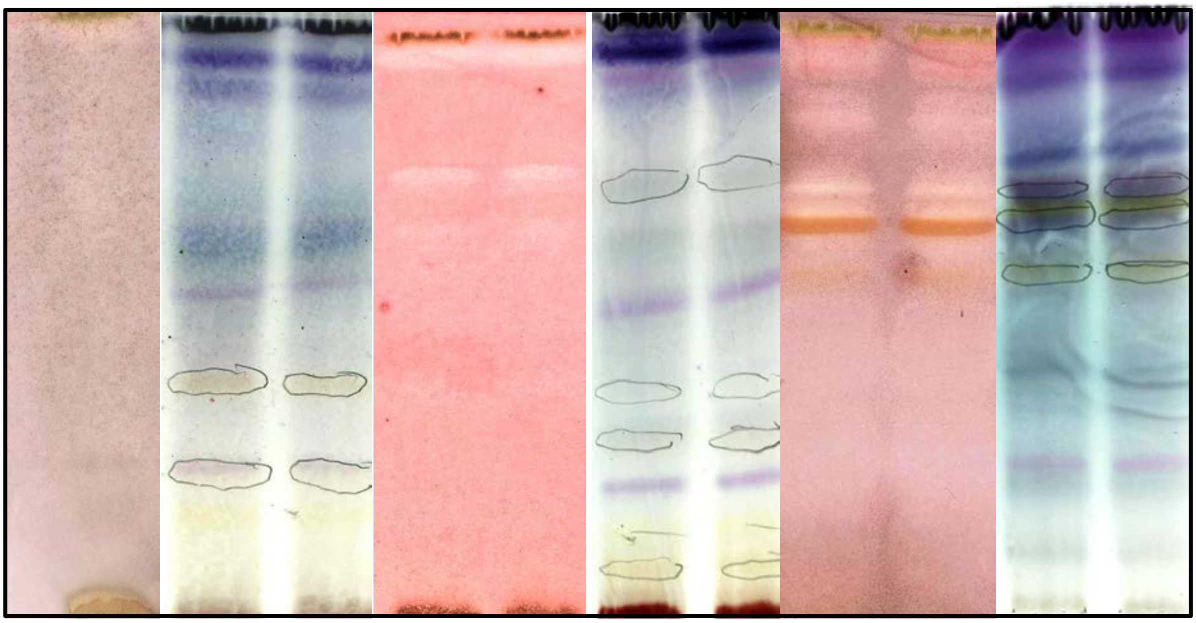
The assay was performed using sterile 96-well microplates with rounded bottoms. The sample to be tested (plant extract, drug or fraction) was prepared at a concentration of 10 mg/mL before serial dilution. One hundred microliters of M7H9 broth or MH broth was added to all the wells then, 100 µl of the sample to be tested were added in three adjacent column wells in the first row whereas other wells included standard drug positive controls to assess bacterial growth, solvent control, broth to be inoculated and clean broth for sterility check. Two samples of crude extract and one standard drug were tested per plate. A two fold serial dilution was carried out leaving 100 µl of different concentrations of diluted tested samples in each well starting with a concentration of 2.5 mg/mL in the first wells. Then 100 µl of the test bacterial suspension were added to all the wells except column 12 (negative control) containing pure broth to make sure that there was no contamination of the broth. Each dose was run in triplicate and the experiment repeated at least six times. The standard antibiotics (reference drugs) included ciprofloxacin, rifampicin, isoniazid and streptomycin starting with a concentration of 100 µg/mL. The microplates were covered and sealed in plastic bags, placed in humid chambers to minimize the evaporation of the culture medium and incubated at 37°C for 24 hours (*M. smegmatis*), 48 hours (*M. fortuitum*) and 72 hours (*M. aurum*). For the pathogenic mycobacteria, microplates were incubated for a period of 7 to 15 days.

At the end of incubation, a volume of 40 µl of 0.2 mg/mL of iodonitrotetrazolium chloride (INT) was added to each well, plates were incubated for 30 minutes or longer at 37°C and the development of colour observed. A coloured red-purple formazan or pink colour indicated the reduction of INT by metabolizing organisms whereas a yellow colour or decrease in colour indicated the inhibition of bacterial growth Eloff (1998b). If the colour development was not strong enough for slow growing organisms, plates were incubated much longer and monitored. The correlation assessing the relationship between the MIC values of the 7 mycobacterial species used (Table 4.3) was calculated using Microsoft Excel 2010 software (Microsoft, Redmond, WA, USA).

4.2.3 Thin Layer Chromatography (TLC) analysis and Bioautography

Ten microliter from 10 mg/mL of relevant extracts (containing 100 µg of extract) was loaded on aluminium-backed TLC plates (Merck silica gel F254) in lines of about 1 cm wide. The TLC plates were eluted in three different mobile solvent systems that included ethyl acetate/methanol/water (EMW) 10:1.35:1, chloroform/ethyl acetate/formic acid (CEF) 10:8:2 and benzene/ethanol/ammonia (BEA) 18:2:0.2

(Kotzé and Eloff, 2002). Chromatograms were examined under ultraviolet light at wavelengths of 254 and 366 nm to visualize UV active compounds that fluoresce and might not be visible after vanillin spray; the plates were then sprayed with a solution made of 0.1 g vanillin in 28 mL of methanol and 1 mL of sulphuric acid (vanillin–methanol-sulphuric acid) and heated at 110°C with a heat gun for colour development and visualization of UV inactive compounds present in the plant extract. More than 10 compounds of different polarities were observed. These compounds were very close to each other in both the solvent systems BEA and CEF. In general, the active plants had different activities against different Mycobacteria. Active compounds included both polar and non-polar compounds based on the R_f values determined in bioautography. In the non-polar solvent BEA, the non-polar compounds travelled some distance up the plate with clear zones of inhibition on plates sprayed with different *Mycobacterium* species whereas in the polar solvent system EMW, non-polar compounds were pushed towards the solvent front and polar compounds off the baseline. The CEF solvent system led to a better separation of some compounds (B in Figure 4.3).



PL (A) PL (B) HC (A) HC (B) SU (A) SU (B)

PL: *Protorhus longifolia*, HC: *Harpephyllum caffrum*, SU: *Searsia undulata*

Figure 4.8: Bioautograms (A) of the three plants extracts with low MIC values and thin layer chromatograms eluted in chloroform/ethyl acetate/formic (CEF) solvent system sprayed with vanillin sulphuric acid (B) showing varied chemical constituents. Bioautogram of the extracts against *Mycobacterium aurum*.

For bioautography, only the rapidly growing organisms were used, namely *M. aurum*, *M. fortuitum* and *M. smegmatis,* because the pathogenic mycobacteria present a high biohazard risk if handled in a spray format. Aerosols of pathogenic species increase risk of possible contamination of the environment and human exposure. Duplicate chromatograms prepared as described above were left uncovered in a dark place and air dried until solvents were completely evaporated for at least 24 hours or overnight. A saturated bacterial culture was centrifuged at 3000 x g for 10 min and the pellet resuspended in fresh appropriate liquid growth medium and prepared at McFarland No 1. The TLC plates were then sprayed with active bacterial suspension (18 to 72 h old cultures depending on the growth rate of the particular *Mycobacterium* species) until completely moist using a glass spray gun linked to a vacuum pump. The moist plates were incubated at 37°C in a humidified chamber under 100% relative humidity for 24 h for *M. smegmatis*, 48 h for *M. fortuitum* and 72 h for *M. aurum*. The plates were then sprayed with 2 mg/mL of INT (iodonitrotetrazolium violet) (Sigma, Johannesburg, South Africa) in distilled water and incubated for a further 30 min to few hours until the development of clear zones against a purple-red background. The emergence of purple-red colour resulting from the reduction of INT into formazan indicated the presence of viable microorganisms whereas clear zones against the purple-red background indicated inhibition of mycobacterial growth indicating the Rf values of antimycobacterial compounds separated on the thin layer chromatography plates (Hamburger and Cordell, 1987).

4.2.4 *In vitro* cytotoxicity assay and selectivity index

The cytotoxicity of crude acetone extracts from plants with good MIC values and clear zones of inhibition on bioautography was determined using a tetrazolium-based colorimetric assay, 3-(4, 5-dimethylthiazol)-2, 5-diphenyl tetrazolium bromide (MTT assay) described by Mosmann (1983) and slightly modified by McGaw *et al*. (2007). Vero African monkey kidney cells were obtained from the cell culture collection of the Department of Tropical Diseases (University of Pretoria), cancer liver cells (hepatoma C3A) were purchased from the American Type Culture Collection (ATCC), Manassas, USA, CRL-10741 and murine macrophage (RAW 264.7) cells purchased from ATCC, ATCC®TIB71™ were used. Cells were cultured in Minimal Essential Medium Eagle with L-Glutamine (EMEM) (BioWhittaker, Lonza, Belgium) supplemented with 0.1% gentamicin (Genta50) (Virbac, Centurion, South Africa) and

5% foetal calf serum (Highveld Biological, Sandton, Modderfontein, South Africa) for Vero cells and 5% sodium pyruvate (Adcock Ingram, Midrand, South Africa) and 10% of foetal calf serum were added for C3A cells. For C3A and Vero cells, a 5 day old confluent culture in 75 cm^2 flasks were harvested and centrifuged at 200 × *g* for 2 min, and the pellet resuspended in growth medium to a density of 0.1 $\times 10^6$ cells/mL whereas RAW 264.7 cells were mechanically scraped, plated at a density of 4×10^5 cells/mL onto 96-well plates containing 100 µL of Dulbecco's Modified Eagle Medium (DMEM) (BioWhittaker, Lonza, Belgium) and incubated in a 37°C, 5% CO_2 incubator for 24 h prior to exposure. The following procedure was the same for all the different types of cells. A sterile 96-well microplate was used; cell suspension (100 µL) was added into each well of columns 2–11. Columns 1 and 12 were used as blanks with 200 µL of growth medium to minimize the "edge effect" and maintain humidity. The plates were incubated for 24 h at 37 °C in a 5% CO_2 incubator and cells were observed until they reached the exponential phase of growth. The cells were then exposed to different concentrations of the extract sample as well as to doxorubicin hydrochloride (Adriblastina CSV, Pfizer, Johannesburg, South Africa) as positive control and acetone as negative control. From a concentration of 100 mg/mL of the extract sample, different concentrations using growth medium as a diluent were prepared, namely 1, 0.75, 0.5, 0.25, 0.1, 0.075, 0.05 and 0.025 mg/mL. The microplates were then incubated for 48 h. After this period of incubation, the medium was removed from the wells containing the cells using a multichannel pipette attached to a pump via a plastic tube; cells were rinsed with 200 µL phosphate buffered saline (PBS, Sigma-Aldrich, Johannesburg, South Africa) and replaced with 200 µL of fresh medium. Then thirty microliters (30 µL) of a 5 mg/mL solution of 3-(4, 5-dimethylthiazol)-2, 5-diphenyl tetrazolium bromide (Sigma-Aldrich, Johannesburg, South Africa) in PBS was added to each well. The plates were incubated for a further 4 h at 37˚C in the CO_2 incubator. After incubation, the medium was carefully removed using the same multichannel pipette connected to a pump without disturbing the MTT formazan crystals in each well. Then 50 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the MTT crystals. Absorbance values were read immediately using a microplate reader (BioTek Synergy HT, Analytical and Diagnostic Products, Johannesburg, South Africa) at a wavelength of 570 nm. Columns 1 and 12 were used as blank. The different concentrations were tested in quadruplicate and the assay was repeated at least three times. The LC_{50} values were calculated as the concentration of plants extracts where 50% of cells were still viable compared to untreated cells in columns 2 and 11. In Table 4.4, the selectivity index of the active plants was calculated using LC_{50} values divided by MIC values (LC_{50}/MIC).

4.2.5 Statistical analysis

All experiments were conducted in triplicate and values expressed as mean ± standard deviation. For the MIC values, re-evaluation of the growth inhibition was conducted where a wide variation within the three experiments was noticed. The relationship between the different mycobacterial MIC values was calculated using the Pearson's correlation coefficient (r).

4.3 RESULTS AND DISCUSSION

4.3.1 Acetone extract plant yield

The extraction yield of the plant species ranged from 0.8 to 18.8% (Table 4.2). The highest percentage was observed with *Searsia magaliesmontana* (18.8%) followed by *Searsia undulata* (12.5%) and *Protorhus longifolia* (8.3%). *Heeria argentea* had the lowest yield of 0.8%. The extraction yield and bioactivities of extracts using different extractants vary strongly (Kotzé and Eloff, 2002), hence the choice of acetone in this study.

Table 4.2: Minimal inhibitory concentration (MIC in mg/mL) and total activity (TA in mL/g) of acetone leaf extract from 15 plants of the Anacardiaceae family against *M. bovis* and *M. tuberculosis*

				MBCG		H37Ra		MBfield		MDR-Tb	
Plant species	Voucher number	Extraction yield	Mass yield (mg)	MIC ± SD	TA	MIC ± SD	TA	MIC ± SD	TA	MIC ± SD	TA
Harpephyllum caffrum	PRU2583	4.6	138	0.11 ± 0.05	1255	0.16 ± 0	863	0.13 ± 0.05	1062	0.21 ± 0.09	657
Heeria argentea	PMDN 751	0.8	24	0.26 ± 0.09	92	0.52 ± 0.18	46	0.26 ± 0.09	92	0.21 ± 0.09	114
Lannea discolour	PMDN 565	5.7	171	0.11 ± 0.05	1555	0.11 ± 0.05	1555	0.21 ± 0.09	814	0.52 ± 0.18	329
Loxostylis alata	PRU3526	4.9	147	0.21 ± 0.08	700	0.24 ± 0.08	613	0.52 ± 0.18	283	0.52 ± 0.18	283
Ozoroa mucronata	NEL71/1993	1.7	51	0.625 ± 0	82	0.625 ± 0	82	0.51 ± 0.17	100	0.51 ± 0.17	100
Ozoroa paniculata	PMDN 568	7.7	231	0.52 ± 0.18	444	0.52 ± 0.18	444	0.51 ± 0.17	453	0.51 ± 0.17	453
Protorhus longifolia	PRU120030	8.3	249	0.11 ± 0.05	2264	0.07 ± 0.02	1186	0.11 ± 0.05	1915	0.11 ± 0.05	2264
Searsia Chirindensis	PMDN 333	3.1	93	0.16 ± 0	581	0.11 ± 0.05	845	0.31 ± 0.01	300	0.26 ± 0.09	358
Searsia leptodictya	NEL94/1969	6.1	183	0.13 ± 0.05	1408	0.42 ± 0.18	436	0.22 ± 0.0.8	832	0.26 ± 0.09	704
Searsia magaliesmontana	NEL68/1997	18.8	564	0.17 ± 0.02	3318	0.17 ± 0.02	3318	0.52 ± 0.18	1085	0.52 ± 0.18	1085
Searsia pyroides	PMDN 570	3.8	114	0.13 ±0.05	877	0.13 ± 0.05	877	0.21 ± 0.09	543	0.21 ± 0.09	543
Searsia undulata	PRU120031	12.5	375	0.07 ± 0.02	3409	0.05 ± 0.02	3409	0.11 ± 0.05	3409	0.11 ± 0.05	2885
Sclerocarya birrea	NEL103/1967	6	180	0.11 ± 0.05	1636	0.11 ± 0.05	1636	0.22 ± 0.08	818	0.22 ± 0.08	818
Smodingium argutum	PMDN 188	3.7	111	0.26 ± 0.09	427	0.26 ± 0.09	427	0.13 ± 0.05	854	0.13 ± 0.05	854
Searsia lancea L	PMDN 750	10.6	318	0.26 ± 0.08	1223	0.26 ± 0.08	1223	0.26 ± 0.08	1223	0.26 ± 0.08	1223
CIP (µg/mL)				0.013 ± 0.006		0.10 ± 0.05		0.013 ± 0.006		>10	
INH (µg/mL				10.42 ± 3.61		2.08 ± 0.90		10.42 ± 3.61		>100	
RIF µg/mL				1.04 ± 0.45		0.26 ± 0.11		1.04 ± 0.45		>100	
Strep µg/mL				>100		0.33 ± 0.11		>100		>100	
Acetone mg/mL				>2.5		>2.5		>2.5		>2.5	

4.3.2 Biological activity of extracts

Bioautography of extracts using two *(M. aurum and M. fortuitum)* out of the three rapidly growing mycobacteria led to strong clear zones of inhibition. This also corresponded with low MIC values obtained ranging from 0.05 to 0.1 mg/mL. Extracts with MIC values ≤ 0.1mg/mL were considered to have significant activity; moderate activity was between 0.1 and 0.625 mg /mL and weak or poor activity with MICs >0.625 mg/mL (Kuete, 2010). All 15 plant extracts had good to moderate antimycobacterial activity against rapidly growing and pathogenic mycobacteria ranging from 0.05 to 0.590 mg/mL. *Searsia undulata* extracts had significant antimycobacterial activity with the lowest MIC value of 0.07 mg/mL for *M. aurum* and *M. fortuitum* followed by *M. smegmatis* with MIC of 0.09 mg/mL. *Protorhus longifolia* extracts also had significant antimycobacterial activity against all three non-tuberculous mycobacteria with MIC values of 0.11 mg/mL for both *M. aurum* and *M. fortuitum* and 0.07 mg/mL for *M. smegmatis*. *Searsia lancea*, *Sclerocarya birrea* and *Harpehyllum caffrum* had moderate activity of 0.420 mg/mL, 0.520 mg/mL and 0.590 mg/mL against *M. aurum* respectively whereas the same plant species had moderate activity with low MIC values of 0.21 and 0.11 mg/mL; 0.13 and 0.21 mg/mL and 0.21 and 0.11 mg/mL against *M. fortuitum* and *M. smegmatis*, respectively. *Searsia undulata* had significant activity against all mycobacteria including *M. bovis* and MDR-TB isolates with MIC ranging from 0.05 to 0.11 mg/mL, and the lowest value of 0.05 mg/mL against *M. tuberculosis* ATCC strain H37Ra.

Positive correlation was observed between the pathogenic isolates, *M. bovis and* MDR-TB*,* with *M. fortuitum* with (r) coefficient correlation values of 0.62 and 0.65 respectively whereas negative correlation with value of -0.005 was observed with *M. aurum*. Good correlation was observed between the ATCC strain, H37Ra and rapidly growing mycobacteria with values of 0.92, 0.87 and 0.37 for *M. fortuitum*, *M. aurum* and *M. smegmatis* respectively and the highest coefficient correlation value of 0.98 between MDR-TB isolate with the pathogenic *M. bovis* isolate (Table 4.3).

Table 4.3: Pearson's correlation coefficient (r) between MIC values of tested Mycobacteria

	Ma	*Mf*	*Ms*	H37Ra	*Mb*	MDR-TB
Ma	1					
Mf	0.62	1				
Ms	0.62	0.20	1			
H37Ra	0.87	0.92	0.37	1		
Mb	-0.005	0.62	0.26	0.34	1	
MDR-TB	-0.06	0.65	0.10	0.35	0.98	1

Ma, *Mycobacterium aurum*; Mf, *Mycobacterium fortuitum*; Ms, *Mycobacterium smegmatis*; H37Ra, *Mycobacterium tuberculosis* ATCC strain 25177; Mb, *Mycobacterium bovis*; MDR-TB, Multi-drug resistant *Mycobacterium tuberculosis.* The values in bold represent the correlation between rapidly growing mycobacteria and pathogenic mycobacteria.

The total activity value of an extract from a plant indicates which species could be the candidates for organic production of active extracts for use by communities because it takes into account not only the MIC of the extract but also the yield of the extract. By dividing the extraction yield in mg/g by the MIC in mg/mL the total activity is calculated in mL/g (Eloff, 2000). This provides the volume in mL that the extract from 1 g of dry material can be diluted and still kills the pathogen. *Searsia undulata* had the lowest MIC values (significant activity) with the highest % yield and highest total activity of 5 357 mL/g on *M. fortuitum* and 2 885 mL/g on MDR-TB followed by *Protorhus longifolia* showing the highest total activity of 3 557 mL/g on *M. smegmatis* and 2 264 mL/g on MDR-TB. The results of total activity using rapidly growing mycobacteria are not shown due to limitation on number of tables. These two plants are the best candidates for further investigation due to their low cytotoxicity and more than 30 antimycobacterial compounds observed using bioautography. In addition, (Mosa *et al*., 2014) reported that *Protorhus longifolia* had antibacterial activity towards resistant clinical isolates of Gram negative (*Pseudomonas aeruginosa*) and Gram positive (*Staphylococcus aureus*) whereas *Searsia undulata* has been used to treat chest cold (Hutchings *et al*., 1996). Specific antimicrobials targeting Gram-negative and Gram-positive bacteria are useful but this does not eliminate the need for broader spectrum antimicrobials. However, other plants with moderate activity are worthy of investigation as studies have shown that there is synergy among different compounds within an extract and once fractionated, different fractions/compounds might be more active than the crude extract (Awouafack *et al*., 2013).

4.3.3 Bioautography and thin layer chromatography analysis

Although all selected plant species had significant to moderate activity against all *Mycobacterium* species tested, plants showing at least MIC below 0.1 mg/mL or close to 0.1 mg/mL for any of the mycobacteria tested were selected to be investigated by bioautography. Five plants were selected namely *Heeria argentea, Lannea discolor*, *Protorhus longifolia*, *Searsia undulata* and *Sclerocarya birrea*. Only three plant species, *Harpephyllum caffrum, Protorhus longifolia and Searsia undulata,* had one or more clear zones of inhibition after spraying with *M. aurum* or *M. fortuitum*. *Searsia undulata* was the only species to show clear zones of inhibition with both *M. aurum* and *M. fortuitum,* with more compounds visible with *M. aurum*. Using bioautography, *M. aurum* was the best indicator of antimycobacterial activity followed by *M. fortuitum*. Low MIC values might not always correlate with visualization of clear zones of inhibition due to the nature of separated compounds on the TLC plate, which may be volatile or experience loss of activity due to the separation of compounds that were acting in synergy in the crude extract. The crude extract of *Searsia undulata* had several active compounds against *M. aurum* – potentially more than ten compounds as some compounds were very close to each other in terms of Rf values ranging from 0.05 to 0.98 in the different solvent systems BEA, CEF and EMW (results not shown). Based on the MIC values and bioautogram patterns, the same plant extracts have shown different activities towards different mycobacteria species. *Mycobacterium smegmatis* also had some low MIC values with few or no visible zones of inhibition. The BEA and CEF solvent systems gave the best separation of compounds at this initial stage (Figure 4.3).

4.3.4 Cytotoxicity assay

Most drugs or other substances are metabolised in the liver and excreted by the kidney. Mycobacterial species causing tuberculosis multiply and hide in macrophages. Therefore, three cell lines were selected to represent these organs namely Vero monkey kidney, human liver hepatoma (C3A) and mouse macrophage cells (RAW 264.7). Cancer cells are abnormal cells, and substances showing cytotoxicity to C3A cells and low cytotoxicity to the Vero cells may also be good candidates for further investigation as anticancer preparations. Cell-based *in vitro* toxicity assays are performed at an early stage of the drug development process in order to remove high-risk materials. Plants extracts showing sensitivity to cell lines with LC_{50} values >0.1 mg/mL are considered not cytotoxic in terms of searching for anticancer compounds (Kuete, 2010).

Crude extracts (*Heeria argentea*, *Lannea discolor*, *Protorhus longifolia*, *Searsia undulata* and *Sclerocarya birrea*) with good antimycobacterial activity (from significant to moderate) and those that showed visible zones of inhibition on bioautograms were selected for cytotoxicity testing (Table 4.4). All crude extracts tested showed low toxicity against all three cell lines (C3A, Vero cells and RAW (264.7) except *Searsia undulata* that had moderate toxicity to C3A cells with LC_{50} of 0.034 mg/mL. This deserves further investigation as source of anticancer substance as C3A cells are cancer cells with abnormal metabolic activity.

The *Searsia undulata* extract had LC_{50} of 0.50 and 0.12 mg/mL on Vero cells and RAW cells respectively with good selectivity indexes of 7.08 on Vero cells and more than 1 on RAW cells for non-tuberculous and MDR-TB mycobacteria. *Protorhus longifolia* had an LC_{50} of 0.62 mg/mL on C3A cells, 0.88 mg/mL on Vero cells and >1 mg/mL for RAW cells with highest selectivity index on Vero cells of 12.6 for *M. smegmatis* followed by 8.02 for *M. aurum*, *M. fortuitum* and MDR-TB. Plant extracts with SI values less than1 mean that the extracts are relatively less toxic to the bacteria and more toxic to the mammalian cells (Eloff, 2000). Therefore, extracts with SI >1 may be relatively safer to use *in vivo* (not accounting for pharmacokinetic parameters) as they are less toxic to mammalian cells but more toxic to the pathogens.

The selectivity indexes, especially of *P. longifolia* with SI = 12.6 could be considered as very promising as a good therapeutic index for a remedy or drug should be ≥10 (Caamal-Fuentes *et al*., 2011). It is also worth noting that efficacy "*in vitro*" might differ from efficacy '*in vivo*" due to different parameters influencing pharmacodynamics and pharmacokinetic of drugs administered by different routes to humans and animals.

In this study, *Protorhus longifolia* and *Searsia undulata* had the highest selectivity index against the three rapidly growing mycobacteria and pathogenic mycobacteria and the highest total activity (Table 4.4). These rapidly growing species had the highest total activity of 5357 mL/g for *S. undulata* and 3557 mL/g for *P. longifolia* which indicates the volume to which the extract from 1 g can be diluted and still inhibit the growth of mycobacteria. It is also worth noting that the leaves of *S. undulata* are chewed by Khoi people to treat chest cold, indicating a potential low toxicity. The mode of administration and interaction with other factors *in vivo* must be considered when assessing toxicity.

Table 4.4: Cytotoxicity (LC_{50} in mg/mL) of extracts and selectivity index against C3A liver cells, Vero kidney cells and RAW 264.7 murine macrophage cells

	SI C3 A					SI Vero cells					SI RAW cells				
Plant species	LC_{50}	MA	MF	MS	MDR-TB	LC_{50}	MA	MF	MS	MDR-TB	LC_{50}	MA	MF	MS	MDR-TB
Heeria argentea	0.314	0.627	1.045	1.206	1.493	0.499	0.998	1.663	1.919	2.375	NT	NT	NT	NT	NT
Lannea discolor	0.408	2.148	2.148	3.139	0.785	NT	NT	NT	NT	NT	>1mg/mL				
Protorhus longifolia	0.621	5.642	5.642	8.866	5.642	0.883	8.024	8.024	12.609	8.024	>1mg/mL				
Searsia undulata	0.034	0.488	0.488	0.380	0.311	0.496	7.083	7.083	5.509	4.507	0.123	1.751	1.751	1.362	1.115
Sclerocarya birrea	0.169	0.326	1.304	0.807	0.770	0.499	0.959	3.836	2.375	2.267	NT				

LC_{50}: Lethal concentration 50, MA: *Mycobacterium aurum*, MF: *Mycobacterium fortuitum*, MS: *Mycobacterium smegmatis*, MDR-TB: Multiple resistant tuberculosis strain, SI: Selectivity index

4.4 CONCLUSION

This is the first report on antimycobacterial activity of these tree species from the Anacardiaceae family in Africa. Acetone leaf crude extracts of all the selected plants have shown significant to moderate antimycobacterial activity. *Harpephyllum caffrum, Lannea discolor, Protorhus longifolia, Searsia undulata and Sclerocarya birrea* subsp. *caffra* had good antimycobacterial activity and low cytotoxicity. Some rapidly growing mycobacteria may be used as indicators for detecting good antimycobacterial activity of plant extracts against pathogenic mycobacteria based on the correlation of MIC values. The best indicators of antimycobacterial activity on MIC and bioautography were *M. aurum* followed by *M. fortuitum*. *Protorhus longifolia* and *S. undulata* had good antimycobacterial activity against all mycobacterial species. Low cytotoxicity was observed on Vero cells and RAW cells. *Searsia undulata* was cytotoxic to hepatoma cells and could be investigated further as a source of potential anticancer substances. Adding to results obtained from the bioautography, MIC values and cytotoxicity as well as ethnobotanical use, *P. longifolia* and *S. undulata* had good potential antimycobacterial activity worthy of further investigation. The ethnobotanical use of chewing *S. undulata* leaves by Khoi people to treat chest colds is interesting and the antimicrobial activity of leaf extracts of this species supports this use although further study is necessary to validate this supposition. Isolation and characterization of compounds from these highly promising species is underway and bioassay-guided isolation of fractions and compounds from *Searsia undulata* forms the topic of the next chapter.

Postscript

In the next chapter 5, preliminary results of two out of the 10 antimycobacterial compounds present in these extracts are provided and spectra obtained are presented in appendix B.

CHAPTER 5:

BIOASSAY-GUIDED ISOLATION OF FRACTIONS AND COMPOUNDS FROM *SEARSIA UNDULATA*

ABSTRACT

The treatment of tuberculosis has been a challenge over the past decade due to the emergence of multi- and extremely drug resistant strains (MDR and XDR). There is an urgent need to find alternative treatments for this deadly and highly contagious disease. *Searsia (Rhus) undulata* (Anacardiaceae) is a medicinal plant; its root has been used in South Africa by the Khoi as an anti-inflammatory. Leaves are chewed to treat chest colds and decoction for post parturient problems. Its crude extracts have shown good antimycobacterial activity in a previous study. Therefore, the aim of this work was to isolate and identify active antimycobacterial compounds. Isolated compounds from *Searsia undulata* were screened for antimycobacterial activity using a twofold serial microdilution assay against fast-growing saprophytic mycobacteria that included *M. smegmatis, M. fortuitum* and *M. aurum.* Cytotoxicity (LC_{50}) was assessed using a colorimetric (MTT) assay against Vero monkey kidney cells. Different fractions, sub-fractions of *Searsia undulata* were prepared using column chromatography and analysed using thin layer chromatography and bioautography to visualize the antimycobacterial activity and isolated compounds. Compounds were identified using MS and NMR spectroscopy. Unknown compound was further processed by melting point and FITR. The chloroform fraction showed more than 5 compounds but only two compounds (SLN1 and PKB) were fully identified respectively as Betulonic acid and a novel compound which did not match with any published data base using SciFinder search tool. *Mycobacterium aurum* and *M. fortuitum* were good indicators of antimycobacterial activity with strong clear zone of inhibition; *M. aurum* showed more than 7 active zones of inhibitions. Chloroform fraction showed more than 5 compounds on TLC, the butanol fraction extracted the least compounds. Betulonic acid and compound PKB had an average MIC of 31.25µg/ml for *M. aurum* and *M. smegmatis* and 23.44µg/ml and 31.25µg/ml for *M. fortuitum* respectively. The highest concentration of

compound tested for cytotoxicity was 200µg/ml and compound PKB was the least toxic out of the two compounds. Several active compounds were observed and correlated with low MIC values. This is the first report on *Searsia undulata* related to antimycobacterial activity and first isolation of the two elucidated compounds. This plant has being investigated further for isolation of the rest of active compounds.

5.1 BACKGROUND

5.1.1 Description and taxonomy

Searsia (Rhus) undulata plant belongs to the old *Rhus* genus also referred to as "Sumac" that was changed to "*Searsia*" (Moffett, 2007). *Searsia undulata* is also called t'kuni (Khoi); Kuni-bush (English) and koeniebos, garrabos (Afrikaans). The genus *Searsia* is heterogeneous in the Anacardiaceae family and comprises more than 200 species (Moffett, 2007). They are found in temperate and tropical regions worldwide. The Anacardiaceae family includes plants that produce gums, resins or latex from which antimicrobial compounds may be isolated.

Searsia undulata is a dense, evergreen shrub or small tree of up to three meters in height native to South Africa (Figure 5.1.a). The leaves are trifoliate (Figure 5.1.b), dark green with a shiny lacquered look arising from a narrow winged leaf stalk. Each leaflet is variable in size and shape, with a pointed tip and a distinctly wavy margin hence the species epithet "undulata". Young leaves are obovate, usually shiny and resinous (very sticky). The inconspicuous greenish flowers (Figure 5.1.b) are followed by small, thinly fleshy, green or slightly reddish berries. *Searsia undulata* has been confused with *R. burchellii* as they are closely related (Palgrave *et al*., 2002). The shrub is widely distributed along the west coast of the Cape and occurs in a wide range of different habitats (Moffett, 1993).

(a) (b)

Figure 5.1: Shrub (a) and trifoliate leaves and greenish flowers (b) of *Searsia undulata*

There are several species distributed in different geographical location. In general, *Rhus* plants can grow in non-agricultural viable regions, and various species have been used by indigenous cultures for medicinal and other purposes (Van Wyk, 2008b).

5.1.2 Traditional use of *Searsia/Rhus* species

Traditionally, the leaves are chewed by the Nama (Khoisan) for chest cold and leaf decoctions for post-partum problems (Fourie and Snyckers, 1984). The roots are used in infective gastrointestinal conditions. Wheat (2014) reported in a survey conducted in Paulshoek community (Namaqualand, Cape Town) that the leaves from *Searsia undulata* called "Taaibos" are used in combination with leaves of "Salie", "balerja" and "koorsbos" as tea against colds and flu whereas crushed leaves mixed with kidney fat are used as an ointment or plaster for boils, ringworms and abscesses. The bark and leaves of several other species are used as medicine and include *R. natalensis, R. burchellii*, *R. chirindensis*, *R. laevigata* and *R. viminalis* (Hutchings *et al*., 1996).

In Kenya and Tanzania, a decoction of boiled roots of *R. longipes* is used for the treatment of indigestion and abdominal pain and inhalation of steam vapour against influenza (Kokwaro, 2009). In East Africa, the boiled extract of fruits of *R. vulgaris* is drunk to stop diarrhoea while the boiled stem extracts are applied to wounds. A mixture of roots with other plants is used in pregnant women to allow easy delivery and it is also used against infertility (Kokwaro, 2009) In China, the decoction of all parts of *R. chinensis* is used for treatment of diabetes mellitus; a decoction from seeds is used for malaria and rheumatism (Djakpo and Yao, 2010; Duke, 1992). Dried fruits of *R. chinensis* are used as infusion to treat diarrhoea, aching gums, toothaches and swollen legs whereas its boiled leaves are used in peptic ulcers, kidney stones, eruptions and bruises (Maina, 2011).

In Turkey, the leaf extract of *R. chinensis* is used in the treatment of animal bites. In North America and India, *R. glabra* is used for the treatment of bacterial diseases such as gonorrhea, urinary tract infections, dysentery, internal and external ulcers and sunburn (Borchardt *et al*., 2008; Hostettmann, 1999; Winston, 2001). This plant is also used to alleviate hot flashes symptoms (Winston, 2001); the American Indians smoke sumac leaves for asthma (Hutton, 2010). The dried fruits ground with salt of *R. coriaria* are used in the Mediterranean and Middle East as a spice and for wound healing (Rayne and Mazza, 2007; Shabbir, 2012). In Tanzania the extract of pounded roots of *R. natalensis* steeped in hot or cold water are used for influenza, abdominal pains, hookworm infestation and gonorrhea; the leaves are used against cough; steam from leaves macerated in hot water are inhaled to fight colds (Maina, 2011).

5.1.3 In vitro biological activities

Research undertaken on *Rhus* extracts to date indicates a promising potential for this genus to provide renewable bioproducts with beneficial bioactivities such as antimicrobial, antifungal, antiviral, antimalarial, antioxidant, antifibrogenic, anti-inflammatory, antimutagenic, antithrombin, antitumorigenic, cytotoxic, hypoglycaemic and leukopenic (Maina, 2011; Rayne and Mazza, 2007)

Much work has been done on the fruits of *R. coriaria*, a spice, which have demonstrated potential antimicrobial and antifungal properties (Ali-Shtayeh *et al*., 2013; Bonjar, 2004; Gabr *et al*., 2014; Gabr and Alghadir, 2015). These properties appear to be ascribed to polar compounds extractable with protic solvents. The stem bark methanol extract of *R quartiniana* was found to have antibacterial activity against gonorrhoea (*Neisseria gonorrhea*) and *Staphylococcus aureus*; anthocyanins, coumarins, essential oils, flavonoids, quinines, saponins, sterols and tannins have been isolated from this plant (Chhabra *et al*., 1987). Biological activities of other *Searsia* species have been reported (Table 4.1 previous chapter).

5.1.4 Isolation of compounds

In Africa, there is no report on isolation of compounds from *Searsia undulata* with antimycobacterial activity, but in general, antimicrobial properties from other species of the genus *Searsia* (*Rhus*) have been reported elsewhere. The favourable worldwide distribution of *Searsia* also suggests that desirable bio-products may be obtained locally with minimal transportation requirements from processing to consumer. However, not all the species studied to date have been fully characterized for isolation of potential antimycobacterial activity. The genus *Searsia* (formerly *Rhus)* is of great interest since the members have been found to possess chemical compounds that are toxic to micro-organisms. These compounds include flavonoids, bioflavonoids, isoquinolines and urushiols among others (Ahmed *et al*., 2001; Chhabra *et al*., 1987; Johns *et al*., 1990; Lin *et al*., 1999; Sebothoma, 2009; Songca *et al*., 2012; Wang *et al*., 2008).

Bioassay-guided fractionation has been used for many years to isolate bioactive natural products. The procedure involves repetitive fractionation of extracts and determining the biological activity of all the fractions (Brusotti *et al*., 2014). The method provides a rational means to isolate bioactive compounds from a complex mixture even though it is consuming and labour intensive (Bero *et al*., 2011; Manvar *et al*., 2012). It has also been found that the bioactive components can be extracted from *Searsia* using environmentally benign solvents that allow for both food and industrial end-uses.

Indeed, antimicrobial compounds active against Gram-positive and Gram–negative bacteria have been isolated from the methanol extract of *Rhus glabra*, a species used in folk medicine in North America. Bioassay-guided fractionation led to isolation of three antibacterial compounds namely methyl ester of 3, 4, 5-trihydroxybenzoic acid (methyl gallate), 4-methoxy-3,5-dihydroxybenzoic acid and gallic acid (Saxena *et al*., 1994). A flavone, apigenin dimethylether extracted from the roots of *S. undulata,* was reported to have anti-inflammatory properties (Fourie and Snyckers, 1984); the root methanol extract from *Rhus succedonea* has been reported to contain compounds such as agathisflavone, robustaflavone, hinokiflavone, volkensiflavone, rhusflavone, succedaneflavone and their methylethers and acetates which were evaluated for antiviral activities. Robustaflavone demonstrated strong inhibitory effects against influenza A and B viruses (Lin *et al*., 1999); the same activity was detected with agathisflavone and amentoflavone which also exhibited moderate anti-Herpes Simplex virus (HSV)-1 and anti-Herpes Simplex Virus (HSV)-2 activities. Anti HIV-1 reverse transcriptase activity has been observed with robustaflavone, hinokiflavone and morelloflavone (Lin *et al*., 1999).

Maina (2011) has reported on bioassay-guided antibacterial activity (using ATCC cultures and clinical isolates of Gram-positive and Gram-negative bacteria) of extracts from root bark, stem bark and leaves of *Rhus natalensis* from which the following compounds were isolated: rhuschromone, chamaechromone, lophirone A, calodenone, rhuschalone II and catechol.

5.1.5 *In vivo* experiments

The National Institute of Health of the United States under the screening programme for new antitubercular drugs recommends that all compounds, potentially antitubercular, be evaluated in both *in vitro* and *in vivo* models. Animal models for *in vivo* evaluation of antimycobacterial activity and toxicity of compounds have been reported (Jiménez-Arellanes *et al*., 2013; Nguta *et al*., 2016; Villar *et al*., 2008). There is no report on the *in vivo* evaluation of antimycobacterial activity/toxicity of *Searsia undulata*.

5.2 MATERIALS AND METHODS

5.2.1 Plant collection and storage

The leaves of *Searsia undulata* were collected during summer (Figure 5.1.a) in November 2013 at the National Botanical Garden in Pretoria, South Africa. Coordinates: 25°44´S, 28°16´E. The tree was identified and labelled in the botanical garden. A voucher number was issued by the University of Pretoria herbarium (PRU) HGWJ Schweickerdt Herbarium, Department of Plant Sciences, where all research specimens are kept. The leaves were air dried at room temperature suspended in bags of net material in a ventilated room (Figure 5.2.b), ground to a fine powder in a Macsalab Mill (Model 2000 LAB Eriez®) (Figure 5.3) and stored in closed jars away from light in the cupboards until used.

(a) (b)

Figure 5.2: Plant collection (a) and air drying of leaves (b)

Figure 5.3: Grinding processes of dried leaves of *Searsia undulata* using a Macsalab Mill

5.2.2 Fractionation of bioactive fractions

5.2.2.1 Bulk extraction and solvent to solvent fractionation

Powder of *Searsia undulata* leaves (1.5 kg) was serially extracted (3 × 4.5 L each) with acetone (Ac). In the first round, the content was left to stand overnight and mixed vigorously on a Labotec shaking machine for 3 h. This process was repeated five times until the colour of the extract decreased. Each time the mixture was vigorously shaken for 2 h on the shaking machine and the extract was filtered using Whatman No. 1 filter paper. For each round, the collected filtrate of the extract was added into a round-bottomed flask and dried using a Büchi R-114 rotary evaporator (Labotec) under reduced pressure (Figure 5.4). The concentrated extract was then poured into a pre-weighed glass beaker and left to dry under a stream of cold air from a wall fan.

Figure 5.4: Crude extract acetone-bulk extraction processes (left) and drying process with a rotary evaporator (right)

A total amount of crude acetone extract (bulk extraction) was obtained (216.06 g). Eighty grams out of 216.06 g extract was taken aside and the rest stored in the fridge. The extract was further processed by fractionating components based on polarity as described (Suffness and Douros, 1979) with minor modifications. The extract (80 g) was dissolved in a 1:1 mixture of chloroform and water and the two phases were separated in a separatory funnel to obtain the chloroform and water fractions. The water fraction was then mixed with an equal volume of *n*-butanol in a separatory funnel to yield the water and *n*-butanol fractions (polar).

On the other hand, the non-polar part included the chloroform fraction which was dried in a round-bottomed flask in a vacuum rotary evaporator and extracted with an equal volume of hexane and 35% of water: methanol mixture to yield the *n*-hexane fraction. Then the chloroform fraction was mixed with 35% water: methanol to yield chloroform and 35% water: methanol fractions.

A bioassay-guided fractionation procedure was followed; collected main fractions (from solvent-solvent fractionation) were tested against rapidly growing mycobacteria and slow growing mycobacteria (pathogenic) to identify the active fractions that will be used for further fractionation. The chloroform fraction, one of the active main fractions, was further processed for fractionation using silica gel columns (Figure 5.5).

5.2.2.2 Column chromatography (CC)

The isolation of the bioactive compounds was carried out using the chloroform fraction with open mobile column chromatography and silica gel as the stationary phase (Figure 5.5). The silica gel powder (90.6 g) was mixed with an appropriate volume of *n*-hexane in a glass beaker in a sonicator and stirred until all bubbles disappeared. The slurry mixture was then poured into a column (50 cm height, 4.5 cm diameter). Extract sample was prepared the previous day: the 9.06 g of chloroform fraction was dissolved in 30 mL of acetone and mixed with 20 g of silica gel and the mixture was allowed to dry under cold air overnight. A filter paper (Whatman no. 1) was cut to fit the inside diameter of the column and was put on top of the silica gel followed by the dried chloroform fraction sample then covered with another filter paper (Figure 5.5).

The solvent system used to elute the different fractions was hexane/ethyl acetate, ethyl acetate/methanol. The sample loaded was adsorbed and eluted starting with 100% *n*-hexane with an increasing amount of ethyl acetate at an interval of 10% until 100% ethyl acetate at the following ratios (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90) and finally 100% ethyl acetate; this was followed by 250 mL volumes of EtOAc-MeOH mixtures using similar ratios until (50:50), then small amounts of MeOH were added in the mixture with EtOAc up to 15% Me(OH) (98.5:2.5), (95:5), 92.5:7.5, 90:10, 95:15). The column was washed with 100% MeOH and the rest of the fractions collected, dried and kept in the fridge. This was done gradually to yield different fractions which were collected into honey jars (250 mL). All the fractions were left overnight to dry in a fume cabinet under a stream of cold air.

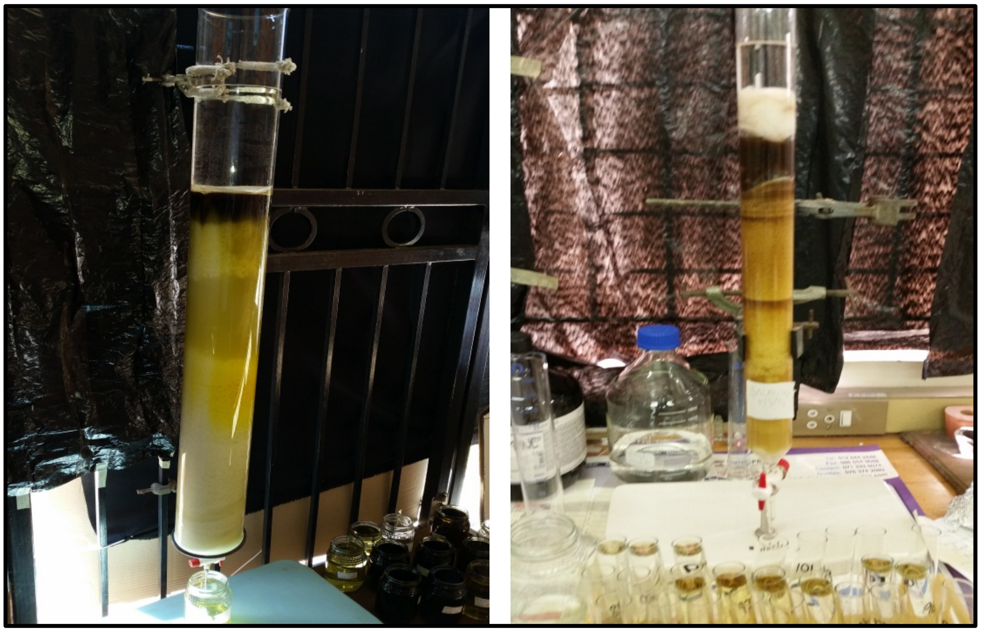


Figure 5.5: Column chromatography used for fractionation

The sub-fractions (F5, F6 and F7) from the chloroform fraction showing the presence of antimycobacterial phytochemical compounds were combined (Figure 5.13-A) and fractionated on a smaller column by gravity elution using hexane and ethyl acetate as solvent system as described above but using a much smaller gradient of 2% to separate compounds of interest. Fractions collected were analysed on TLC and three compounds were isolated. Later on, more of the chloroform fraction stock (33 g and 140 g) was processed as described above; targeting the same fractions based on Rf values, bioautography and TLC fingerprint patterns with the aim of collecting a larger amount of the compounds of interest for further analysis.

5.2.3 Thin layer chromatography (TLC) fingerprinting and bioautography of fractions and compounds

The composition of fractions and compounds was determined by TLC using aluminium-backed sheets of 20 X 20 cm (TLC Silica gel 60 F_{254}, Merck, Germany). Ten microliters from 10 mg/mL of selected fractions dissolved in acetone (containing 100 µg of extract) were loaded on TLC plates in lines of about 1 cm wide using a micropipette. The lines were allowed to dry at room temperature in the dark. Mobile phases of varying polarities were used to develop the plates, namely hexane/ethyl acetate at different ratios [6:4], [7:3], [8:2], [8.5:1.5], [8.8:1.2], chloroform/ethyl acetate/formic acid (CEF) [10:8:2] and benzene/ethanol/ammonia (BEA) [18:2:0.2] (Kotzé and Eloff, 2002). The above procedure, using the crude extracts of *Searsia undulata,* has been reported in Chapter 4 in section 4.2.3. More than ten compounds of different polarities were observed. These compounds were very close to each other in both the solvent systems BEA and CEF. Active compounds included both polar and non-polar compounds based on the R_f values determined on bioautograms. In the non-polar solvent BEA, the non-polar compounds travelled some distance up the plate with clear zone of inhibition on plates sprayed with different mycobacteria species. The intermediate solvent system CEF led to a better separation of some compounds (Figure 4.3 previous chapter).

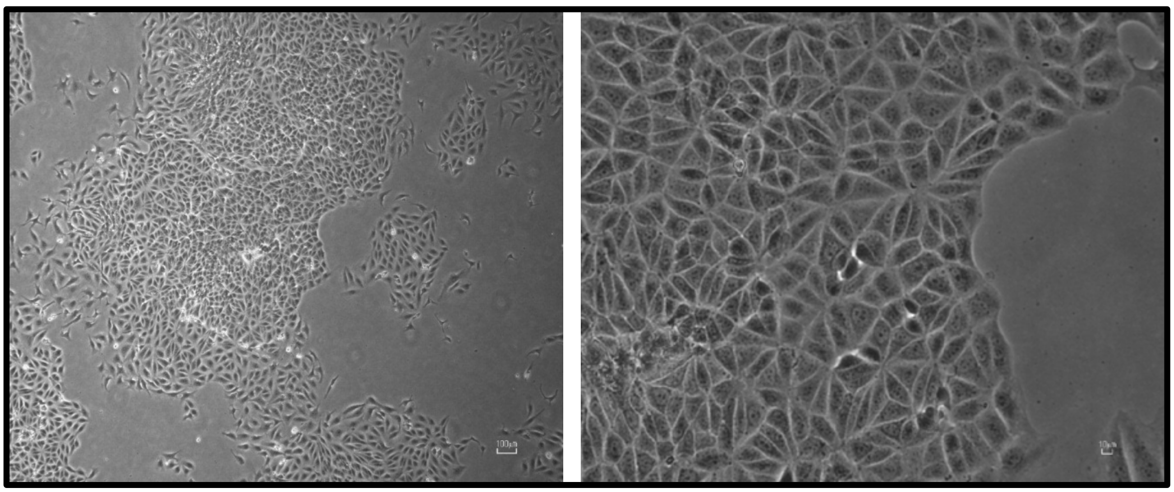
For bioautography, only the rapidly growing organisms were used, namely *M. aurum*, *M. fortuitum* and *M. smegmatis,* because the pathogenic mycobacteria present a high biohazard risk if handled as a spray. Mycobacteria used were cultured and maintained as described in Chapter 4 under section 4.2.2.3. Duplicate chromatograms prepared as described above were left uncovered in a dark place and air-dried until solvents were completely evaporated for 24 h or overnight. A saturated bacterial culture was centrifuged at 3000 x g for 10 min and the pellet resuspended in fresh appropriate liquid growth medium and diluted to a McFarland No. 1. The chromatograms were then sprayed with active bacterial suspension (18 to 72 h old cultures depending on the growth rate of the particular *Mycobacterium* species) until completely moist using a glass spray gun linked to a membrane pressure pump. The moist plates were incubated at 37°C in a humidified chamber under 100% relative humidity for 24 h for *Mycobacterium smegmatis*, 48 h for *M. fortuitum* and 72 h for *M. aurum*. The plates were then sprayed with 2 mg/mL of INT (Sigma-Aldrich, Johannesburg, South Africa) in distilled water and incubated for a further 30 min to few h until the development of a purple-red background. The emergence of purple-red colour resulting from the reduction of INT (Iodonitrotetrazolium violet) into formazan indicated the presence of viable microorganisms whereas clear zones against the purple-red background indicated inhibition of mycobacterial growth indicating the R_f values of antimycobacterial compounds separated on the TLC plates (Hamburger and Cordell, 1987).

5.2.4 Minimum inhibitory concentration (MIC) of *Searsia undulata* fractions and compounds

The method was carried out as described in Chapter 4, section 4.2.2.4. All the fractions comprised a mixture of phytochemical constituents and were tested at 10 mg /mL whereas the concentration for the purified compounds was 1 mg/mL. The MIC of extracts, fractions and compounds was determined at each fractionation stage.

5.2.5 Cytotoxicity of compounds

The cytotoxicity assay was carried out as described in Chapter 4, section 4.2.4 and Vero monkey kidney cells were used (Figure 5.6).



(a) (b)

Figure 5.6: Normal Vero monkey kidney cells viewed under the microscope at magnifications of 10X (a) and 40X (b)

5.2.6 ^{13}C and 1H nuclear magnetic resonance (NMR)-procedures and structural elucidation of isolated compounds

5.2.6.1 Isolation and characterization of compounds

Glass columns (20-25 mm diameter) wet packed with silica gel 60 (0.040-0.063 mm) 230-400 Mesh ASTM, Merck) were used for column chromatography, and elution was done with hexane, ethyl acetate, MeOH and chloroform (CHCl3). Isolation of compounds from the combined semi-purified fractions, F5, F6 and F7 (Figures 5.14-A to 5.15-B) was carried out using a small column packed with silica gel with 100% n-hexane and fractions were collected into honey jars. Hexane is a non-polar solvent that extracts mostly non-polar alkaloids, triterpenes, phenolics, flavonoids, steroids and volatile oils (Adeshina *et al*., 2012).

5.2.6.2 Sample preparation of compounds

Samples of compounds were submitted to the Department of Chemistry at the Council for Scientific and Industrial Research (CSIR) and the University of Pretoria, South Africa for structure elucidation.

5.2.6.2.1 Compound SLN1

The sample SLN1 was dissolved in 200 µL d_6-acetone (500 µL ampoule, 99.99% deuterated acetone, C_3D_6O, Merck) and immediately transferred to a new 7″ Norell Select Series 3 mm i.d. NMR tube and sealed for analysis.

5.2.6.2.2 Compound PK-B

The sample was partially soluble in acetone; but soluble in DMSO-d6 which was then used as a solvent. DMSO (200 µL) was used to dissolve the sample from the vial and transferred immediately to a new 7″ Norell Select Series 3 mm i.d. NMR tube and sealed for analysis.

5.2.6.3 NMR spectrometers and structure elucidation

5.2.6.3.1 Compound SLN1

Two spectrometers were used to accomplish the acquisitions, namely a Varian Premium Shield VNMRS triple resonance system (Direct Digital Receiver 1) operating at 600 MHz, and a Varian INOVA dual band system operating at 400 MHz. The 600 MHz system was equipped with a 5 mm room temperature H{CN}probe, and was used to acquire the proton spectrum of the material as well as all two dimensional spectra. The 400 MHz system was equipped with a 3 mm room temperature dual resonance indirect. Both systems were operated using Agilent VNMRJ 4.2A software.

Compounds at CSIR were measured on a Varian-600 NMR machine (Agilent Technologies, California). Chemical shifts were recorded in σ (ppm). All spectra were processed on ACD Labs Structure Elucidator 2015 software. All signals in both one-and two dimensional spectra were individually hand-selected to ensure signal purity and alignment.

5.2.6.3.2 Compound PKB

The mass spectrum was acquired by Synapt G2 high definition mass spectrometry in positive ESI mode. The NMR spectra were recorded on a 400 MHz Bruker AVANCEIII NMR spectrometer at 25^{o}C. The chemical shifts from ^{1}H NMR and ^{13}C NMR spectra are reported in parts per million relative to the solvent residual peak. The chemical shifts used for referencing ^{1}H and ^{13}C Spectra were 2.50 ppm and 39.5 ppm, respectively. The coupling constants (*J*) were measured in Hz. Abbreviations used for the multiplicity of signals are as follows: singlet (s), doublet (d) and broadened (br).

5.3 RESULTS AND DISCUSSION

5.3.1 Bulk extraction, fractionation, thin layer chromatography and bioautography

5.3.1.1 Extraction yield, thin layer chromatography and bioautography

The 1.5 kg finely ground *S. undulata* leaves yielded 216.06 g of crude acetone extract (Figure 5.7). Eighty grams out of 216.06 g was processed using solvent-solvent fractionation and yielded five fractions, namely chloroform, hexane, 35% water: methanol, butanol and water based on polarity which varied widely (Figure 5.8). The highest mass was obtained in the intermediate polarity chloroform fraction (53.27 g) with only 0.34 g in the non-polar hexane fraction. The butanol fraction contained 9.2 g, the water fraction 3.75 g and the 35% H_2O in methanol 0.34 g. more polar (intermediate polarity). The yield of each fraction obtained after solvent-solvent fractionation of the bulk acetone extract (80 g) is presented in Figure 5.8.

From the 80 g of acetone crude extract processed, the total mass recovered was 66.9 g which means about 84% recovery. The 16% loss might have been due to the presence of pellicles that could not be separated and to the sticky, oily nature of the extract which was not easy to recover during solvent-solvent fractionation; the oily supernatant observed when separating hexane from chloroform fractions was filtered out. The percentage of recovery for each fraction was respectively 79.6% for chloroform, 13.75% for butanol, 5.6% for water and 0.51% for hexane and 35%H_2O/methanol. With 0.5 % of recovery for the non-polar fraction (hexane) and 80% of intermediate polarity (chloroform), the results from the different solvent systems showed compounds of intermediate polarity and variable active compounds from non-polar to polar. The TLC fingerprint of the butanol fraction did not contain visible compounds after vanillin spray and colour development but some compounds were visible under UV light and those compounds were more visible on bioautograms (Figures 5.9-5.11-C)**.**

Initially 55 fractions were collected and analysed on TLC plates using hexane: ethyl-acetate at ratio 7:3 and 6:4, fractions with similar profiles were combined into 12 sub fractions (F1-F12) (Figures 5.7, 5.12-5.13). Fractions collected with 100% methanol are not included in figure 5.7 as they had no antimycobacterial activity.

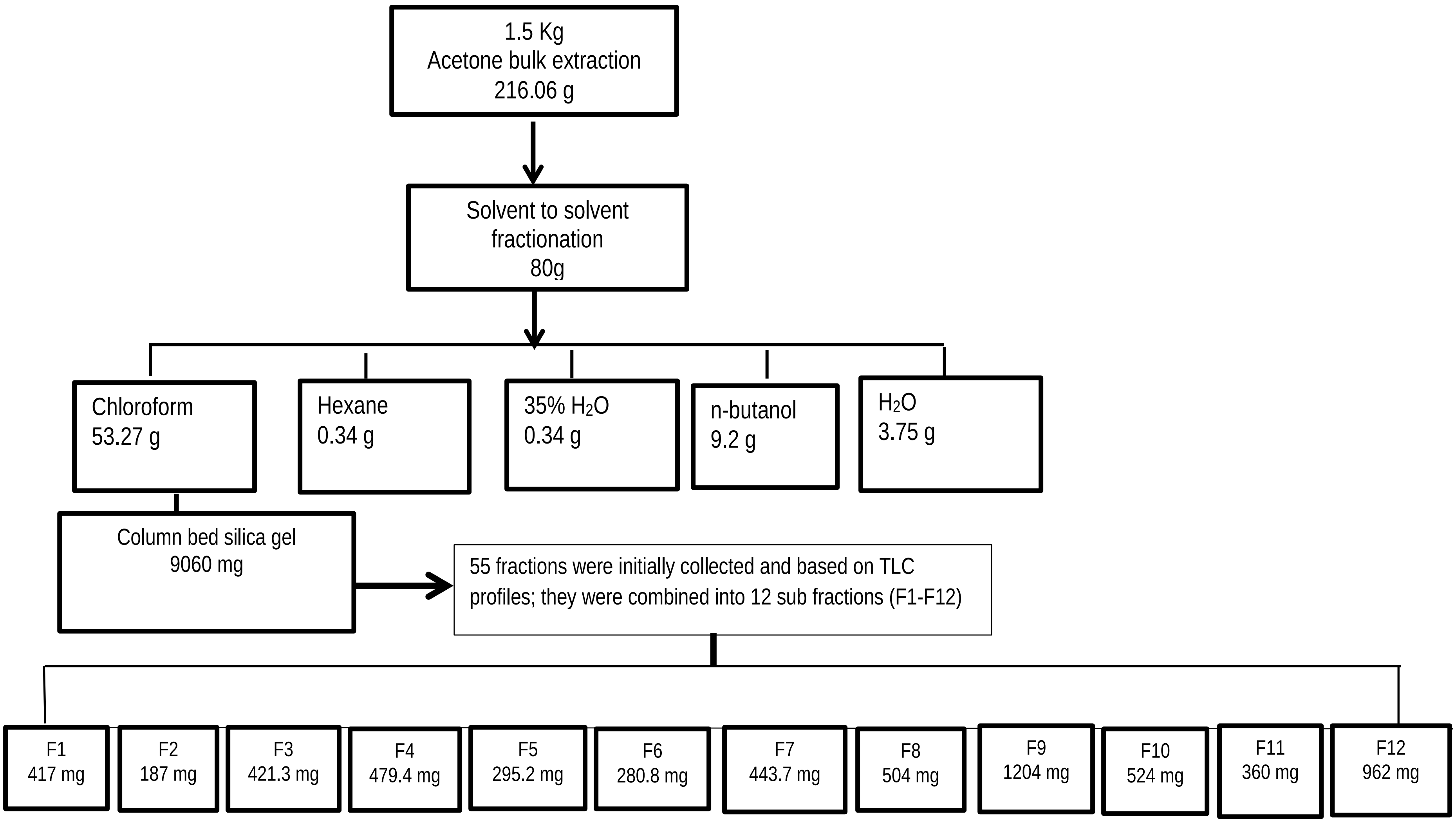


Figure 5.7: Schematic extraction and fractionation results towards compound isolation from *Searsia undulata*

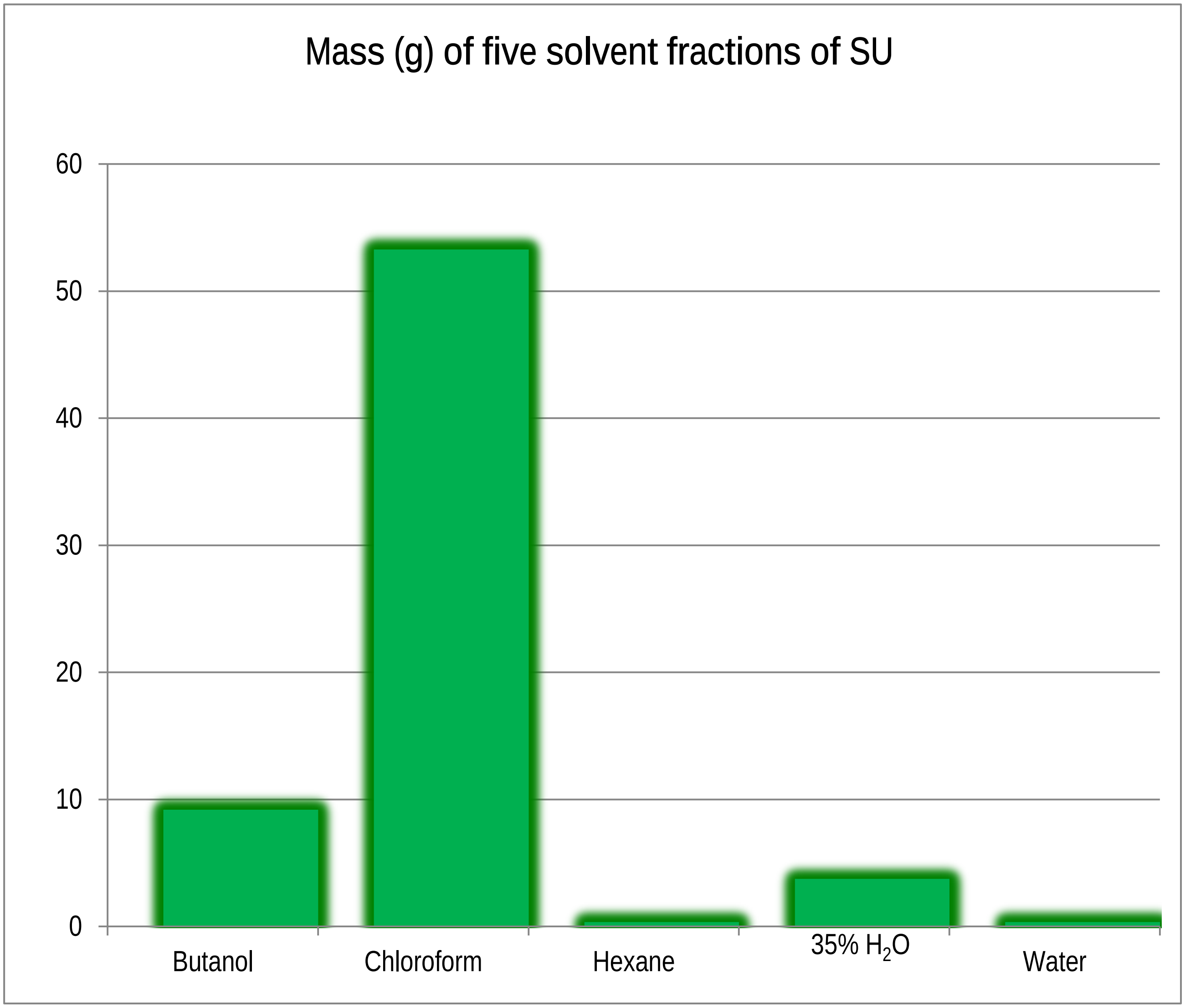


Figure 5.8: Mass (g) of the five solvent-solvent fractions obtained from 80 g of S. *undulata* leaf extracts

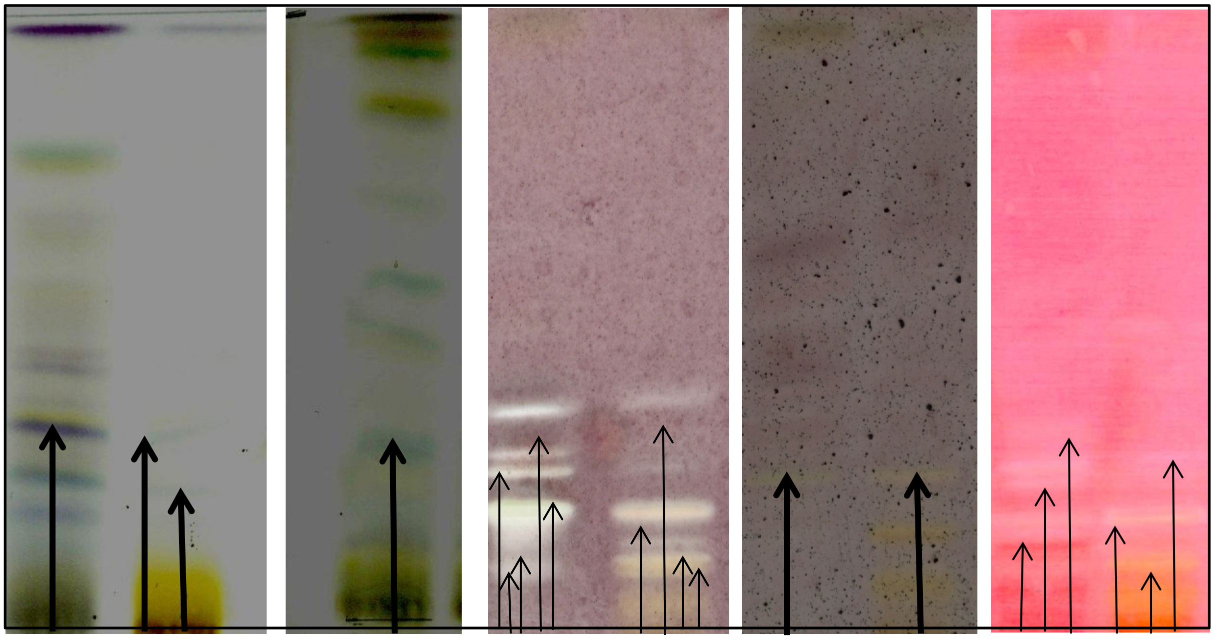


Figure 5.9: Chloroform and butanol fractions- TLC fingerprinting and bioautograms of plates eluted in BEA and sprayed with rapidly growing mycobacteria spp. CHL: Chloroform, BUT: Butanol, HE: Hexane, E: Ethyl acetate, MA: *M aurum*, MF: *M fortuitum*, MS: *M smegmatis*

The solvent-solvent fractions with high yield, good MIC values and presence of antimycobacterial compounds in bioautograms were selected (chloroform, butanol and 35% water fractions) and tested further to determine antimycobacterial activity against *M. aurum*, *M. fortuitum* and *M. smegmatis* (Figures 5.10 A - 5.11 C). Antimycobacterial activity was observed for all three mycobacteria although MIC values were different. The difference in MIC may be due to the difference in concentration of inactive compounds in different fractions or to synergism. Based on the bioautograms the most active compounds observed were non-polar and did not separate well. The chloroform fraction was selected for further work and fractionated by silica gel column chromatography then sequentially eluted with increasing polarity of n-hexane/ethyl acetate mixture to yield 55 fractions. The chemical composition was determined by TLC and fractions were combined based on similar composition (Figures 5.10 - A, 5.11 - A.1) and bioautograms (Figures 5.12-5.13). The results indicated that most compounds were non polar; indeed a better compound separation was observed in the BEA solvent separation system.

5.3.1.2 TLC fingerprinting and bioautography of fractions and compounds

5.3.1.2.1 Solvent-solvent fractions

The five fractions were eluted in BEA solvent system and showed the presence of similar compounds. The acetone crude extracts (ACE) showed similar compounds when compared to *n*-hexane (HE) and chloroform (CHA, CHB) fraction. The chloroform and hexane fraction profiles were more similar showing a better separation. The more polar water fraction, 35% water fraction in methanol (35% H_2O), did not separate well in the non-polar eluent system as expected. But, this fraction showed two compounds similar to those present in the *n*-hexane and chloroform fractions. The *n*-butanol and water fractions did not show any visible compounds. The water fraction was not further processed as no compounds were observed on vanillin-sprayed TLC plates and bioautogram; the hexane fraction was similar to chloroform fraction. The butanol fraction was tested and showed clear zones of inhibitions on bioautograms although there were almost no visible compounds on vanillin-sprayed TLC plates eluted in the same solvent system BEA (Figures 5.9 - 5.11-A1).

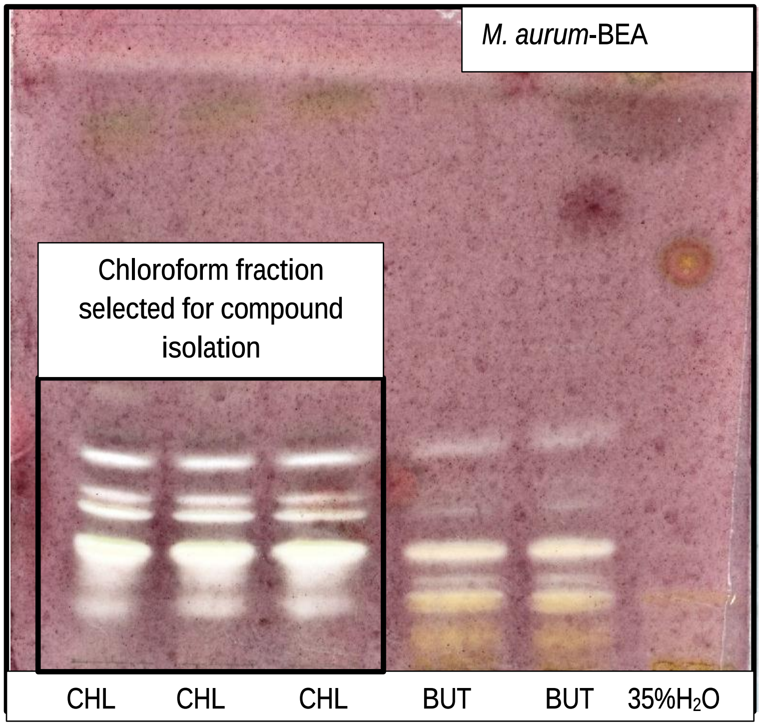


Figure 5.10-A: Bioautogram of *M. aurum* eluted in BEA (Benzene/ethanol/ammonia at ratio 18:2:0.2) separated in triplicate (CHL) and sprayed with 2 mg/mL of INT (Iodonitrotetrazolium violet) showing solvent-solvent fractions of chloroform (CHL), butanol (BUT) and 35% water (H_2O) of SU

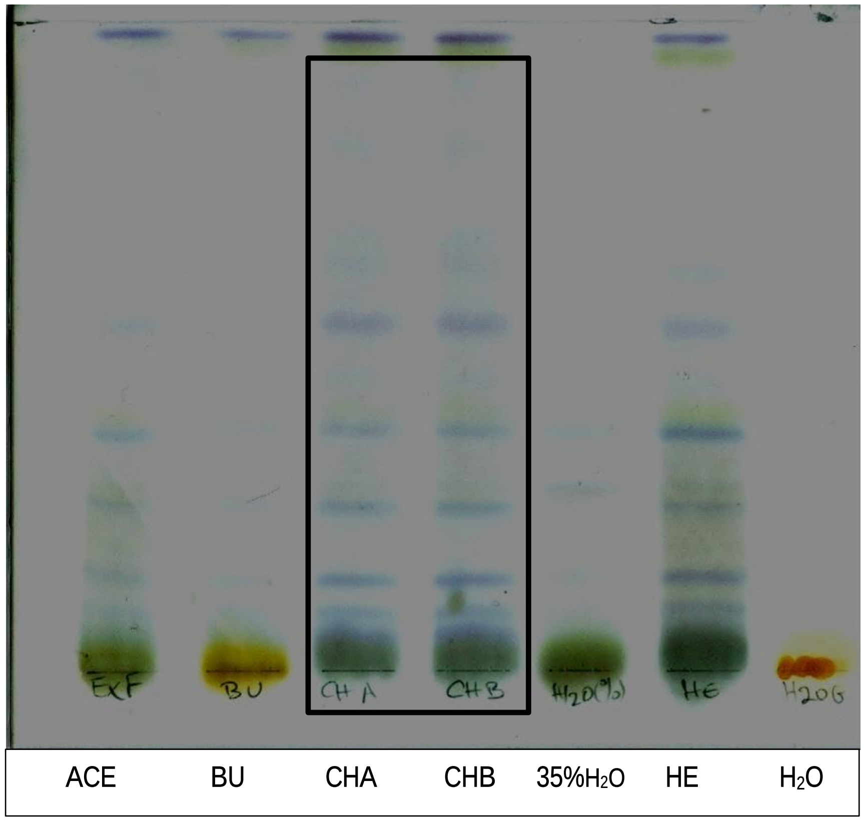


Figure 5.11-A1: TLC fingerprint of acetone crude extract and solvent - solvent fractions (Butanol, chloroform, 35% water, hexane and water)

The phytochemical analysis and bioautograms of the solvent to solvent fractions eluted in the different mobile solvent systems and sprayed with the different mycobacteria species, namely *M. aurum*, *M. smegmatis* and *M. fortuitum* are shown as plates sprayed with *M. aurum* eluted in BEA (Figure 5.14-A1) and CEF (Figure 5.14-B); plates with *M. fortuitum* eluted in BEA and CEF solvent (Figures 5.15-A and 5.15-B) then the plates with *M. smegmatis* in BEA and CEF (Figures 5.16-A and 5.16-B). The non-polar fractions separated well in the non-polar eluent system. All fractions, namely chloroform, *n*-butanol and 35% water fraction showed in bioautography the presence of compounds inhibiting the growth of mycobacteria species although the TLC fingerprints of butanol did not show the presence of visible compounds.

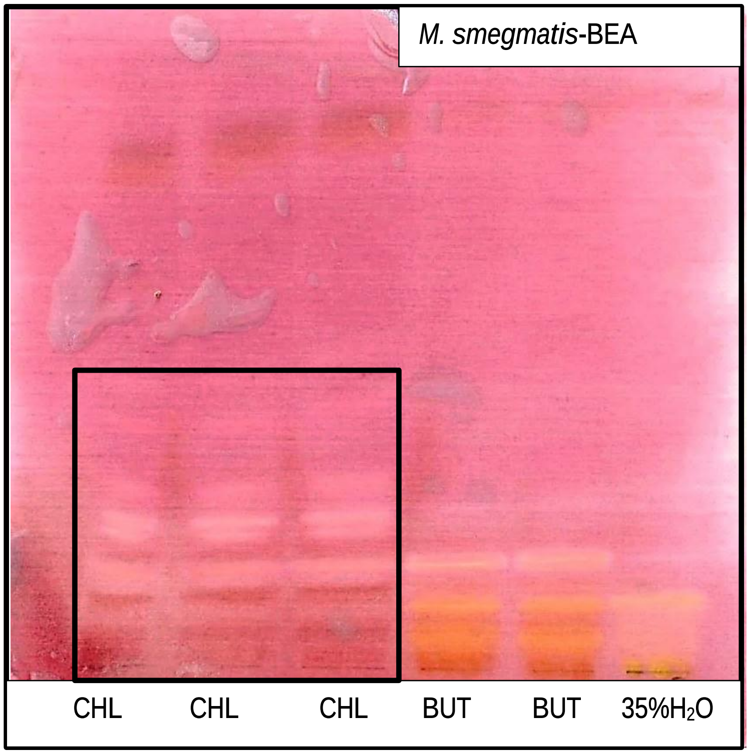


Figure 5.11-B: Bioautogram plate eluted in BEA (Benzene/ethanol/ammonia at ratio 18:1:0.2) and sprayed with *M. smegmatis* followed by INT indicating antimycobacterial compounds of solvent-solvent fractions CHL: chloroform, BUT: butanol and 35% H_2O: 35% water in methanol of SU. Clear zones indicate mycobacterial growth inhibition

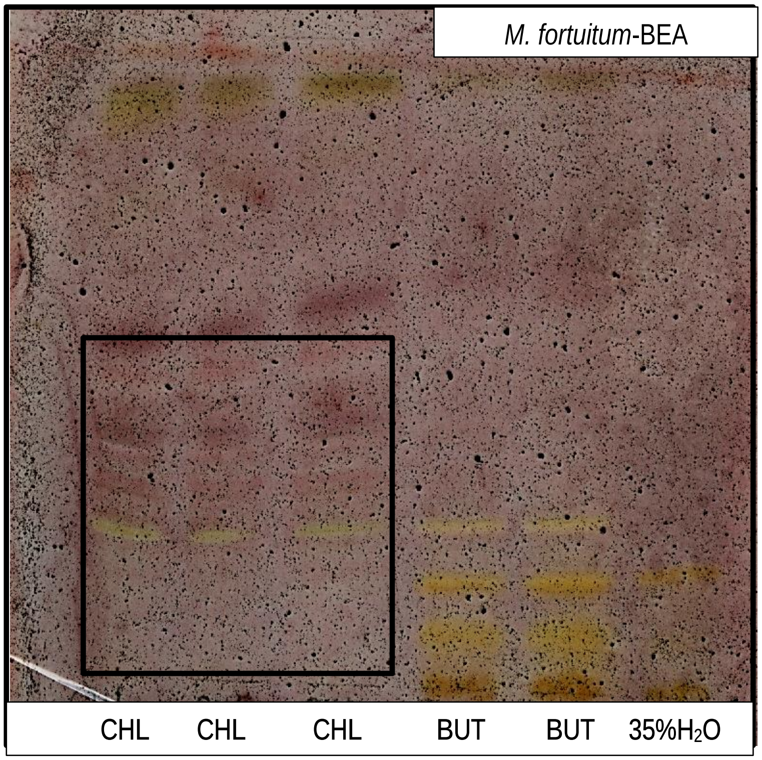


Figure 5.11-C: Bioautogram plate eluted in BEA (Benzene/ethanol/ammonia at ratio 18:2:0.2) and sprayed with *M. fortuitum* and followed by INT indicating antimycobacterial compounds of solvent-solvent fractions CHL: chloroform, BUT: *n*-butanol and 35%H_2O: 35% water in methanol of SU. Clear zones indicate mycobacterial growth inhibition

The isolation of the compound was bioassay-guided based on the presence of antimycobacterial activity observed as clear, white zones of inhibition on the bioautograms at each fractionation stage. Most compounds were non-polar and close to each other which made the process of separation laborious (Figures 5.10 A – 5.11 C).

Based on the high yield of the chloroform fraction (53.27 g) and strong antimycobacterial activity observed on the bioautography and MIC values, the chloroform fraction was selected. An amount of 9.06 g was used for further fractionation towards isolation of the compound in an open column on silica gel. Fifty five fractions were collected in different jars at the initial fractionation; these fractions were combined into 12 fractions (F1-F12) and tested as previously described with *M. aurum* and *M. smegmatis* (Figures 5.12, 5.13, 5.14 and 5.16).

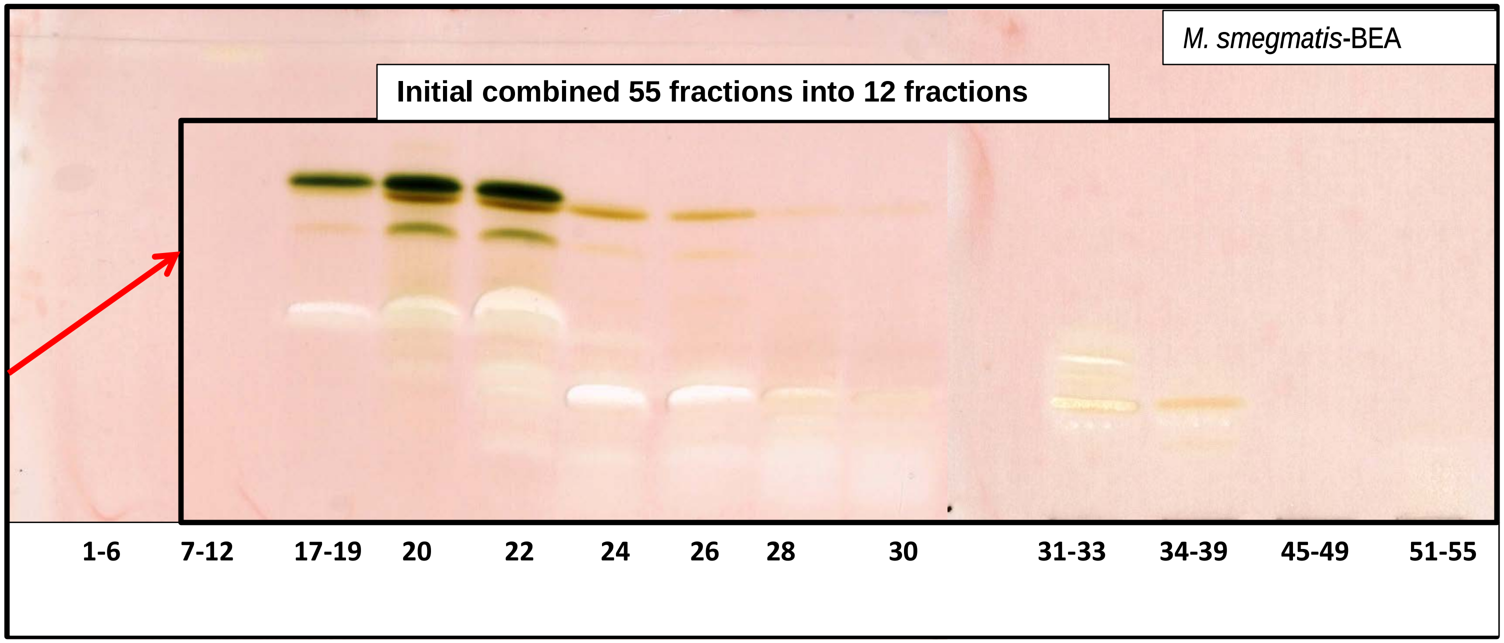


Figure 5.12: Fractions (n=55) collected from the chloroform fraction were combined, tested and based on clear zone of inhibition pattern against *M. smegmatis*, combined into 12 semi-purified fractions (F1-F12) that were separated further for compound isolation

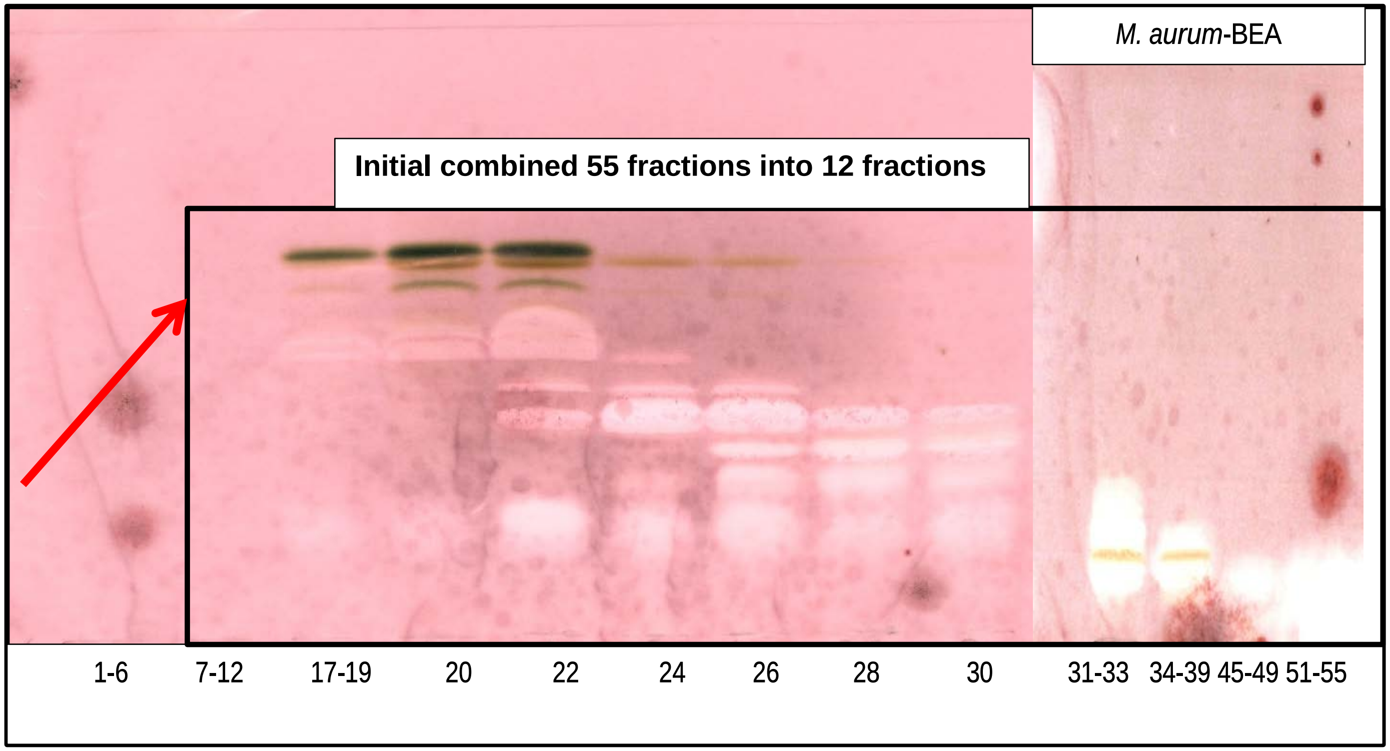


Figure 5.13: Fractions (n=55) collected from the chloroform fraction were combined, tested and based on clear zone of inhibition pattern against *M. aurum*, combined into 12 semi-purified fractions (F1-F12) that were separated further for compound isolation

5.3.1.2.2 TLC fingerprinting and bioautograms of sub-fractions

The chloroform fraction (9.06 g) yielded 12 fractions amounting to 6.0784 g which is about 67% recovery. The percentage of recovery below 80% represented the portion of fractions containing most of the targeted antimycobacterial compounds as shown on the different figures, R_f and MIC values. Three out of the 12 sub-fractions, F5, F6 and F7, represented 16% of the collected sub-fractions (1.0197 g) (Figure 5.7). The sub-fractions F5 to F7 showed similar consistent patterns of strong clear zones of inhibition against all mycobacteria species tested - *M. aurum*, *M. smegmatis* and *M. fortuitum* - on plates eluted in BEA and CEF (Figures 5.14 to 5.16). Adding to good MIC values obtained, they were combined and processed further for the preliminary isolation of some of the compounds (Figures 5-17, 5.18-5.19).

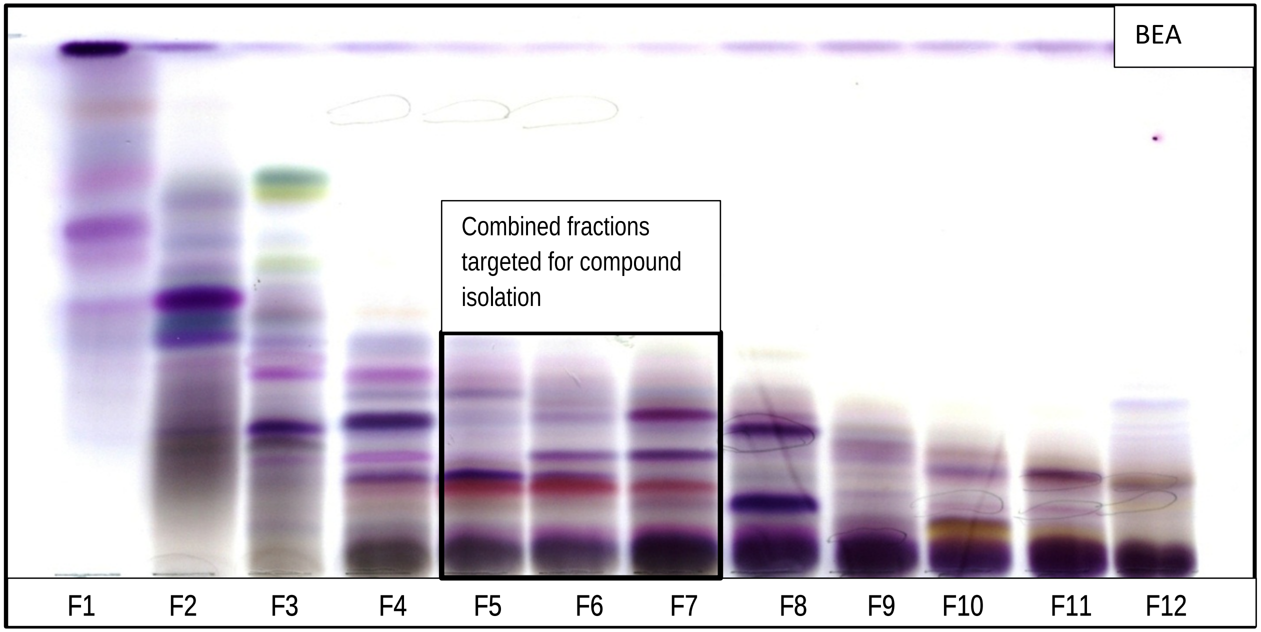


Figure 5.14-A: TLC fingerprints of the 12 pooled fractions obtained from the chloroform fraction; plate was eluted in BEA (Benzene/ethanol/ammonia at ratio 18:2:0.2). F5 to F7 fractions targeted for compound isolation

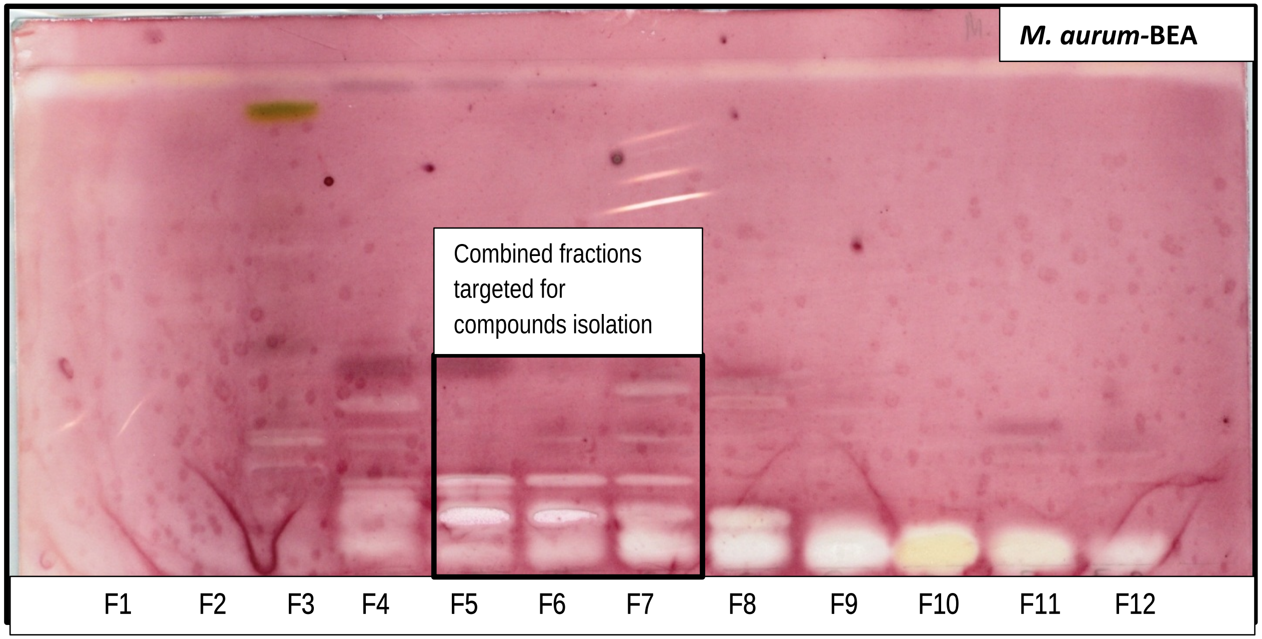
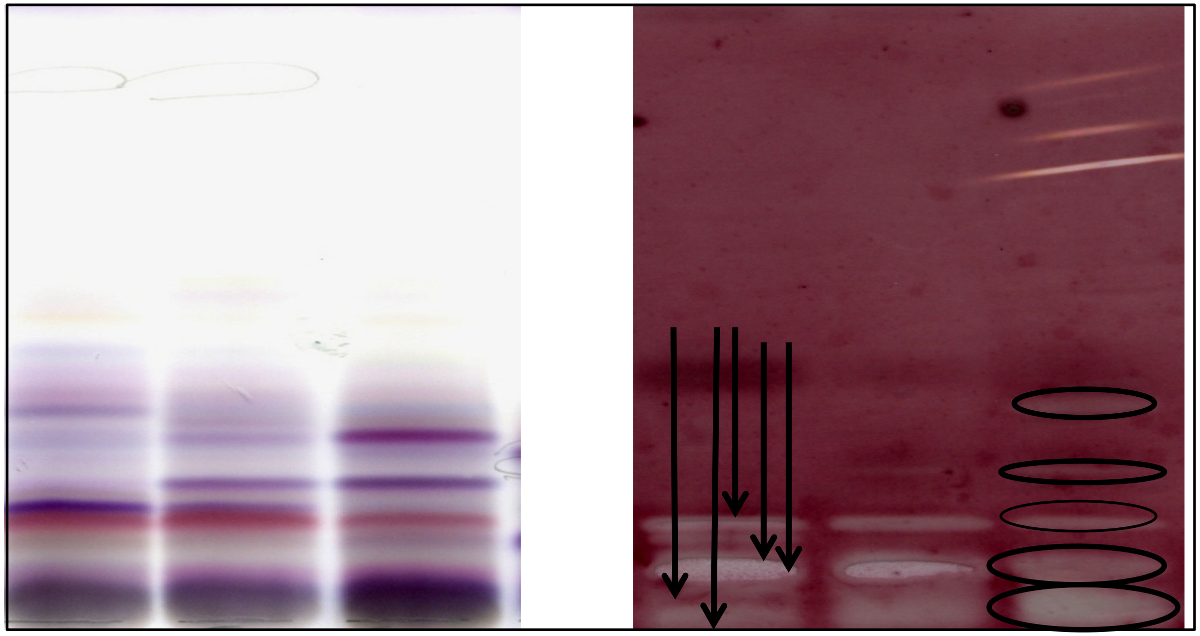


Figure 5.14-A1: Bioautogram eluted in BEA sprayed with *M. aurum* followed by INT indicating consistent clear zone of inhibition with focus on fractions F5, F6 and F7

The different zones of inhibition appearing as white bands represent the lack of growth of the targeted *Mycobacterium sp*. These zones were rated according to the intensity of their appearance on a scale of 1 to 5 as visible bands observed on bioautograms. Scale 1 representing a weak intensity and scale 5 a strong intensity. Their R_f values were also calculated to locate the active chemicals in the elution solvent CEF as non-polar compounds were targeted (Tables 5.1, 5.2 and 5.3). In the intermediate CEF solvent system, low polarity compounds tend to have higher R_f values and are located higher on the TLC plate (Figures 5.14-B, 5.15-B and 5.16-B). The intensity of some of the fractions corresponded to the lowest MIC values. The presence or absence of zones of inhibition also varied from one *Mycobacterium* sp. to the other, confirming that the susceptibility of an antimicrobial varies from one microorganism to the other.



(a) (b)

F5 F6 F7 F5 F6 F7

Figure 5.14-A2: Vanillin sprayed TLC plate and bioautogram plate eluted in BEA (Benzene/ethanol/ammonia at ratio 18:2:02) sprayed with *M. aurum* followed by INT indicating the three pooled fractions (F5, F6 and F7) that were separated further for isolation of compounds indicating the closeness of the active compounds

Table 5.1: The intensity of the zones of inhibition on a five point scale and their R_f values as observed in Figure 5.14-B

	Rated Intensity of zone of inhibition against *M. aurum* on bioautography and Rf values in CEF solvent								
Rf values	F4	F5	F6	F7	F8	F9	F10	F11	F12
0.95	3	2	2	2					
0.90-0.92	5	5	4	5	5	5	2		
0.88-0.89		3	2	5			4	2	
0.85-0.87					5	5	4	4	
0.81-0.83		4	3	2	3	5	4	5	5
0.8									
0.71-0.73						5			
0.68-0.69	1	2	2	1			4	5	5
0.64-0.67						5	5		5
0.61									

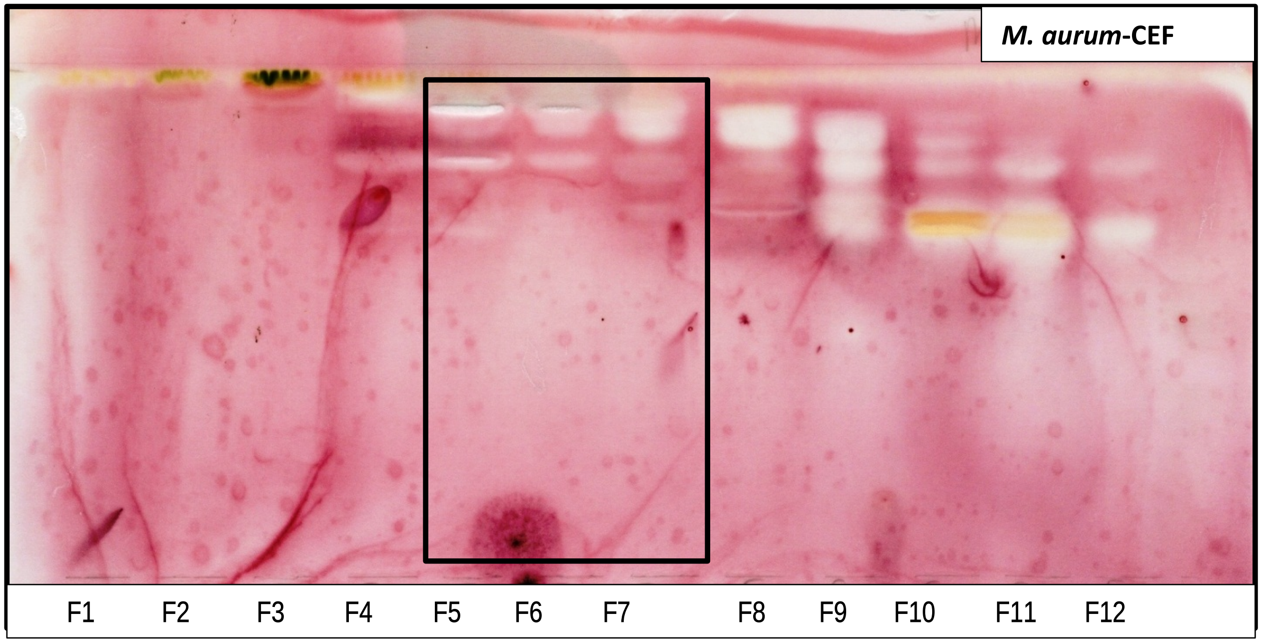


Figure 5.14-B: Bioautogram eluted in CEF (Chloroform/ethyl acetate/formic acid at ratio 10:8:2) sprayed with *M. aurum* followed by INT indicating 12 pooled fractions from the chloroform with consistent clear zone of inhibition focusing on fractions F5, F6 and F7. The clear zones indicate mycobacterial inhibition growth

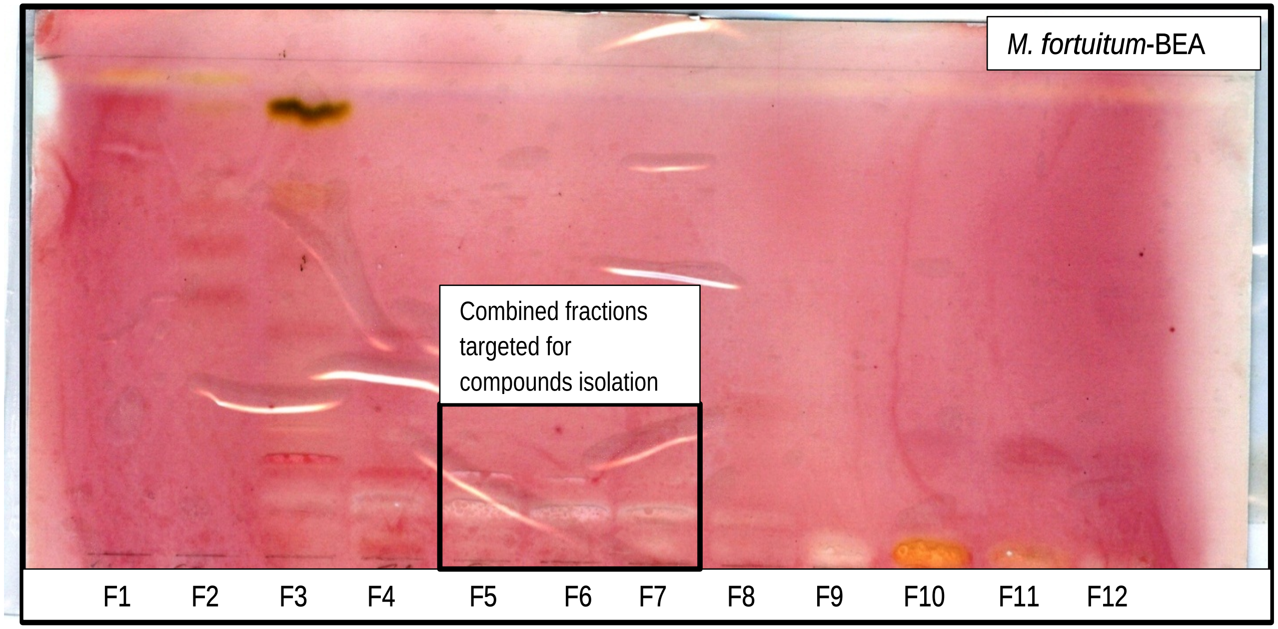


Figure 5.15-A: Bioautogram eluted in BEA sprayed with *M. fortuitum* followed by INT indicating 12 pooled fractions from chloroform with zones of growth inhibition focusing on fractions F5, F6 and F7

Table 5.2: The intensity of the zones of inhibition on a five point scale and their R_f values as observed in Figure 5.15-B

	Rated Intensity of zone of inhibition against *M. fortuitum* on bioautography and R_f values in CEF solvent								
Rf values	F4	F5	F6	F7	F8	F9	F10	F11	F12
0.95									
0.90-0.92	1	1			5				
0.88-0.89	2	5	5	5	5	5			
0.85-0.87				5					
0.81-0.83	2	4	3		3				
0.8						5			
0.71-0.73				2		4	1	1	
0.68-0.69							1	2	
0.64-0.67	2	3	2		5	4			
0.61									

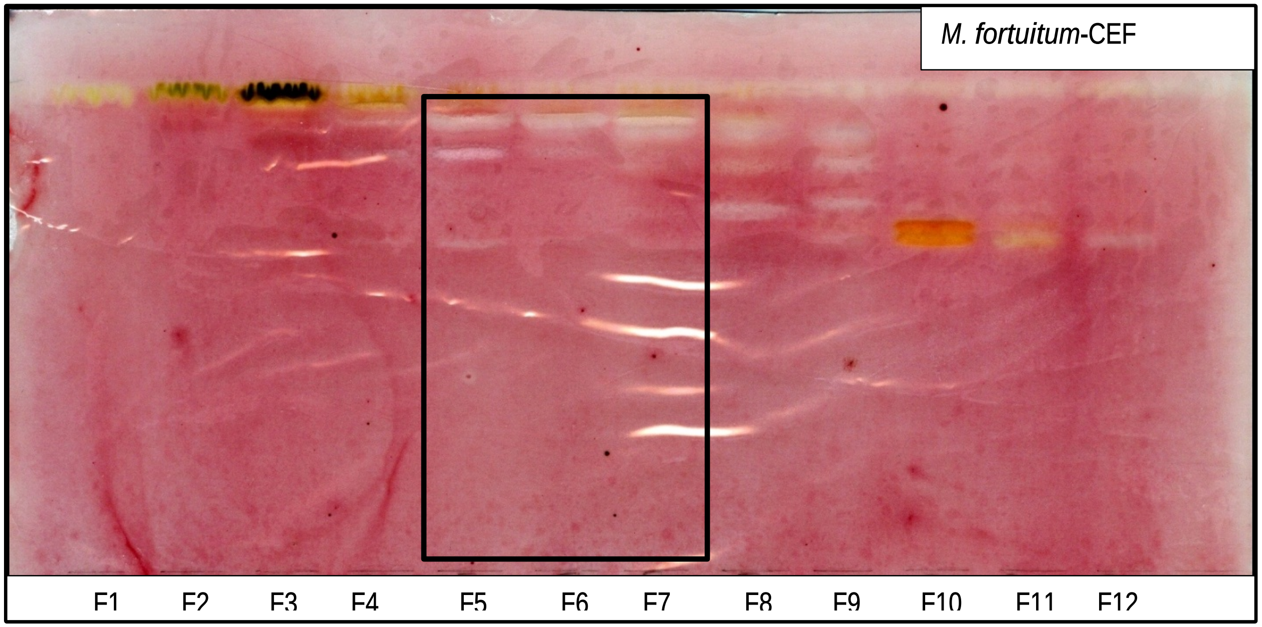


Figure 5.15-B: Bioautogram eluted in CEF and sprayed with *M. fortuitum* followed by INT indicating 12 pooled fractions from chloroform with zones of growth inhibition focusing on fractions F5, F6 and F7

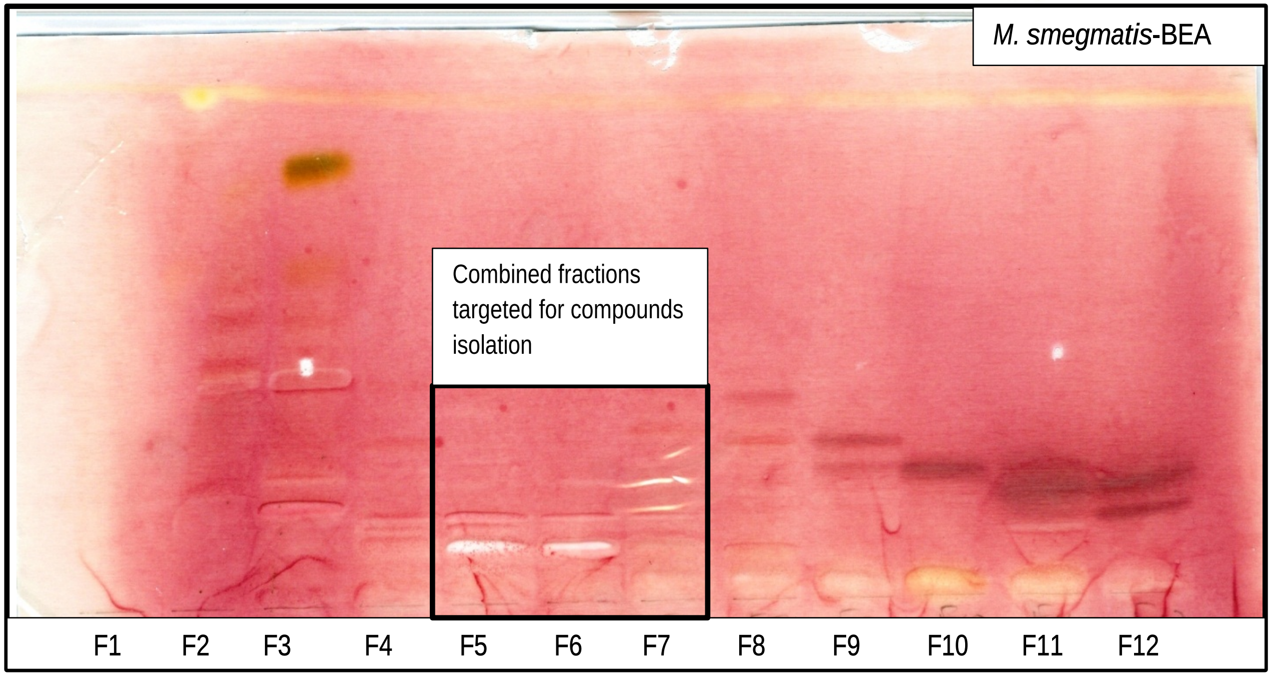


Figure 5.16-A: Bioautogram eluted in BEA and sprayed with M. smegmatis followed by INT indicating 12 pooled fractions from chloroform with zones of growth focusing on fractions F5, F6 and F7

Table 5.3: The intensity of the zones of inhibition on a five point scale and their R_f values as observed in Figure 5.16-B

	Rated Intensity of zone of inhibition against *M. smegmatis* on bioautography and R_f values in CEF solvent								
Rf values	F4	F5	F6	F7	F8	F9	F10	F11	F12
0.95									
0.90-0.92	3	1							
0.88-0.89									
0.85-0.87		5	5	5	5				
0.81-0.83	4				2	2			
0.8						2			
0.71-0.73									1
0.68-0.69									
0.64-0.67							2	2	1
0.61							4	4	3

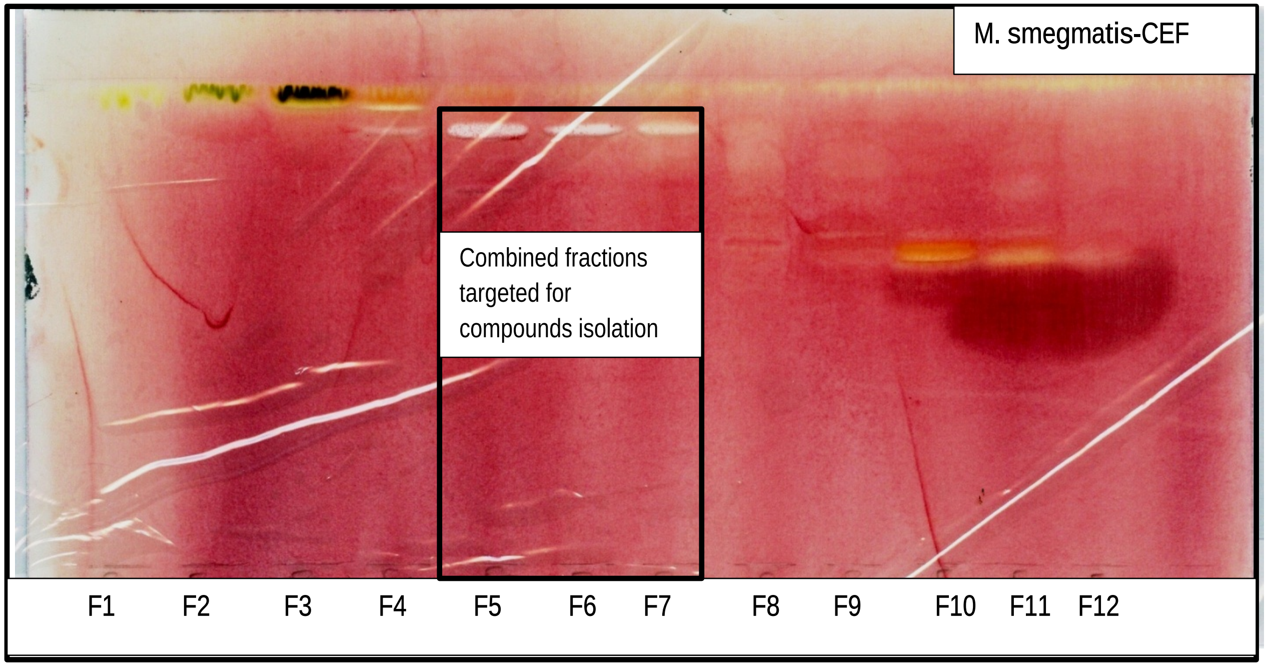


Figure 5.16-B: Bioautogram eluted in CEF and sprayed with *M. smegmatis* followed by INT indicating zones of growth inhibition focusing on fractions F5, F6 and F7

Two plates were eluted in the BEA solvent for each fraction and sprayed, one with vanillin and the other with the mycobacterial suspension broth of *Mycobacterium aurum*. The plates were placed next to each other to visualize the compounds inhibiting the growth of mycobacteria appearing as white zones against a reddish background indicating the growth of the mycobacteria. Prior to spraying the TLC plates with either vanillin or mycobacteria, they were observed under UV light at 254 nm and 366 nm to visualize any fluorescent compounds invisible to the naked eye. Two fluorescent compounds were observed (Figure 5.17). Fungal contamination during the period of this research was also a challenge which affected the quality of some plates as dark pinpoints or stains, but without jeopardizing the results.

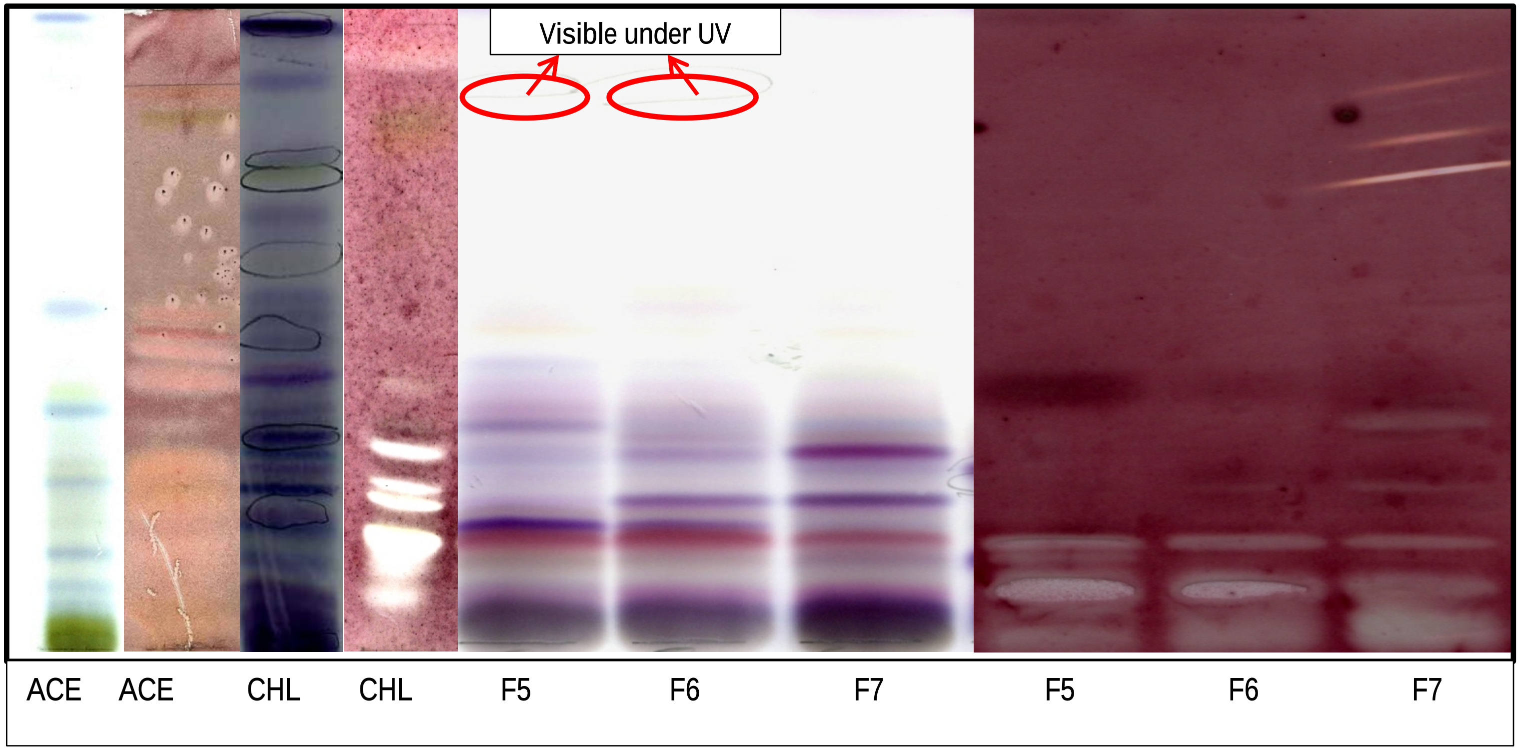


Figure 5.17: TLC fingerprints and bioautograms showing the different stages of isolation from the acetone crude extract to fractions of *Searsia undulata.* Plates were eluted in BEA, TLC plates were sprayed with vanillin and bioautograms with *Mycobacterium aurum* (which was one of the best indicators of mycobacterial activity) followed by INT. ACE: Acetone crude extract; CHL: Chloroform fraction; F5-F7: sub-fractions obtained from 55 fractions from the chloroform fraction

5.3.1.2.3 Sub-fractions and compounds

Based on the MIC, TLC fingerprints, bioautograms and total activity of each fraction, three fractions were selected (F5, F6 and F7) to target compounds with the best antimycobacterial activity (Figures 5.14 - 5.16). Two fluorescing compounds were observed under UV light at 366 nm wavelength (Figure 5.17) but did not show activity on bioautography and were not targeted for isolation during the period of this study.

The three sub-fractions (F5-F7) showed strong clear zones of inhibition and they were compared to crude extracts, solvent to solvent fractions and compounds. Observation on the separation of the fractions of interest from crude extract to isolation of compounds is easy to follow (Figures 5.17-5.18). The bioautogram plate was sprayed with *M. aurum* which was one of the best indicators of antimycobacterial activity. The chloroform fraction showed a clear zone of inhibition of a compound that is more polar and this compound seemed not to show inhibition when fractionated further. This could be explained that further separation did take place and the fraction lost the active portion as the synergistic interaction of compounds within the fractions might be lost during the separation process. Based on R_f values, the same compounds were present in all the fractions.

The TLC fingerprint of the chloroform fraction indicated the presence of more than 20 potential fractions whereas the crude extract showed about 10 visible fractions, which confirms that the process of fractionating was indeed taking place. In general, active fractions were close to each other as shown on all the profiles (Figures 5.9 - 5.11). The isolated compounds were only tested using the MIC assay and for cytotoxicity. More work is still to be done which includes testing against pathogenic mycobacteria, synergy with current standard anti-TB drugs and isolation and identification of more compounds while assessing the stability of fractions and compounds in different environmental conditions. Some of the fractions deposited crystals on standing while others remained in the form of an oily paste. The bioactivity of the crude acetone extract of *Searsia undulata* has been reported in Chapter 4, but results of the crude extract are shown and used to discuss results obtained from the fractions and compounds (Figure 5.18). The 55 fractions obtained from the chloroform fraction of SU were tested against *M. aurum*, *M. fortuitum* and *M. smegmatis* and their antimycobacterial activity determined on bioautography observed as clear zones of inhibition (white zones). The results revealed that fractions 5, 6, and 7 (F5, F6 and F7) had the consistent inhibitory activity against all three mycobacteria species and indicated similar compounds. Other fractions contained one or two active compounds that were not consistent with the different intensity of the clear zones of inhibition for all three tested mycobacteria. Observations made on bioautograms eluted in BEA and sprayed with *M. aurum* revealed that F3 had 3 visible clear zones of inhibition which were also present in F4 but F4 showed two visible active phytochemicals of which neither was present in F3 or F5, F6 and F7. Fractions F8 to F12 showed a similar active phytochemical but did not show the same compounds as observed in F5 to F7. F5, F6 and F7 were therefore selected for further isolation based on consistency observed (Figures 5.14 – 5.18).

Compounds were eluted with 100% hexane with increasing polarity with 2% ethyl acetate resulting in sequential elution of SLN1 (26.2 mg); PK-A1 (15.4 mg), PK-B (22.4 mg), PK-C (15.9 mg) and PK-D (8.4 mg). PK-B was obtained by eluting the compound with a mixture of chloroform and methanol (Figures 5-18-5.19).

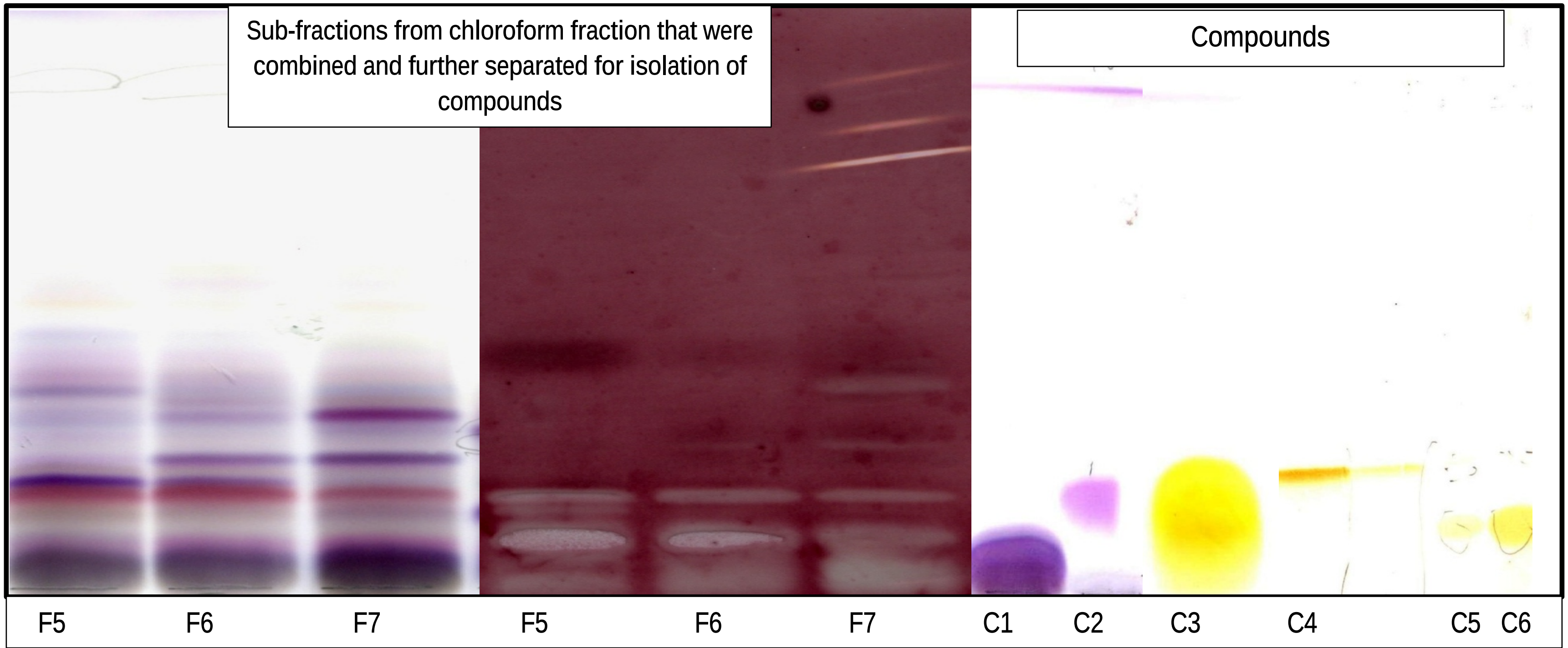


Figure 5.18: TLC fingerprints and bioautograms indicating targeted fractions F5-F7 to compounds isolated from *Searsia undulata.* Bioautogram were eluted in BEA solvent systems and sprayed with *Mycobacterium aurum* which was one of the good indicators of mycobacterial activity. TLC plates for compounds were eluted in different ratios of hexane: ethyl acetate and sprayed with vanillin

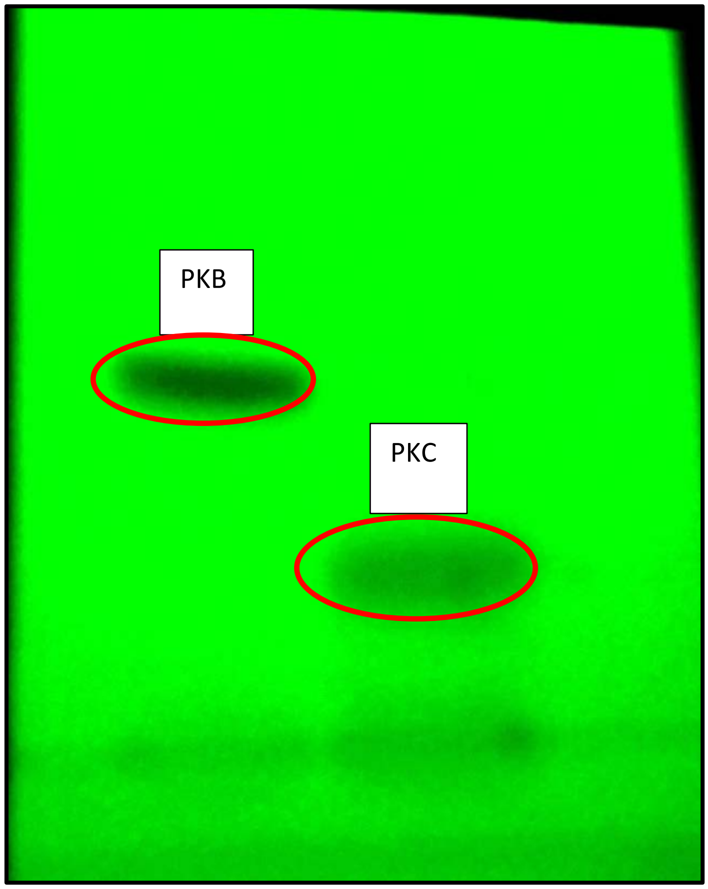


Figure 5.19: TLC plate visualized under UV light (366 nm) showing the separation of compound B from compound C during the purification process

5.3.2 Minimum inhibitory concentration of fractions and compounds

5.3.2.1 Minimum Inhibitory concentration of fractions

The bioautography profile of the fractions achieved during the isolation of compounds as shown in the previous section showed different patterns of clear zones of inhibition against the different mycobacterial species used, namely *M. aurum*, *M. fortuitum* and *M. smegmatis*. This corresponded with good MIC values ranging from 0.058 to 0.468 mg/mL.

The MIC assay was carried out as previously described against selected organisms. The antimycobacterial activities as MIC values were reported for the crude extract of *Searsia undulata* were reported in Table 4.2 in the previous chapter. The MIC obtained for each of the 12 fractions is shown in Table 5.4. Fractions with MIC values below 0.1 mg/mL were considered to have significant activity; moderate activity was between 0.1 and 0.625 mg/mL and weak activity >0.625 to 2.5 mg/mL or poor activity >2.5 mg/mL (Kuete, 2010).

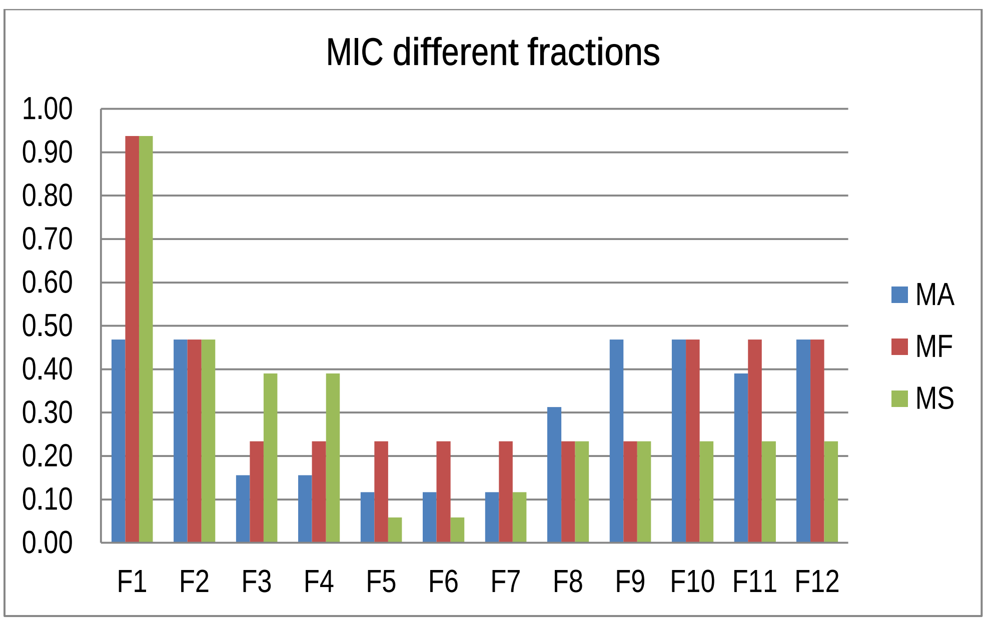


Figure 5.20: The MIC value (mg/mL) of each fraction against the different mycobacteria. MA: *M. aurum*, MF: *M. fortuitum*, MS: *M. smegmatis*

Fractions that included main fractions obtained from solvent to solvent fractionation and sub-fractions collected after elution of the chloroform fraction in the open column silica gel chromatography had good to moderate activity that did not always correlate with the presence of clear zones of inhibition on bioautograms.

The fractions F5, F6 and F7 had significant antimycobacterial activity showing similar compounds and hence were selected for further column chromatography with MIC values of 0.117 mg/mL for *M. aurum*, 0.234 mg/mL for *M. fortuitum* and 0.058 mg/mL for *M. smegmatis* for all fractions except F7 with MIC value of 0.117 mg/mL for *M. smegmatis.* Fraction F5 and F6 had the lowest MIC of 0.058 mg/mL observed against *M. smegmatis*. The lowest value of MIC, the more effective is the fraction at a lower dosage whereas 1/MIC represent the sensitivity of the organism tested, the highest the value of 1/MIC, the more sensitive is the organism tested against the specific fraction. The MIC values of each fraction against all three mycobacteria species, sensitivity of each mycobacteria and the overall view of each fraction's average activity is presented in Figures 5.20, 5.21 and 5.22. Fractions F5, F6 and F7 were active against all three mycobacteria species.

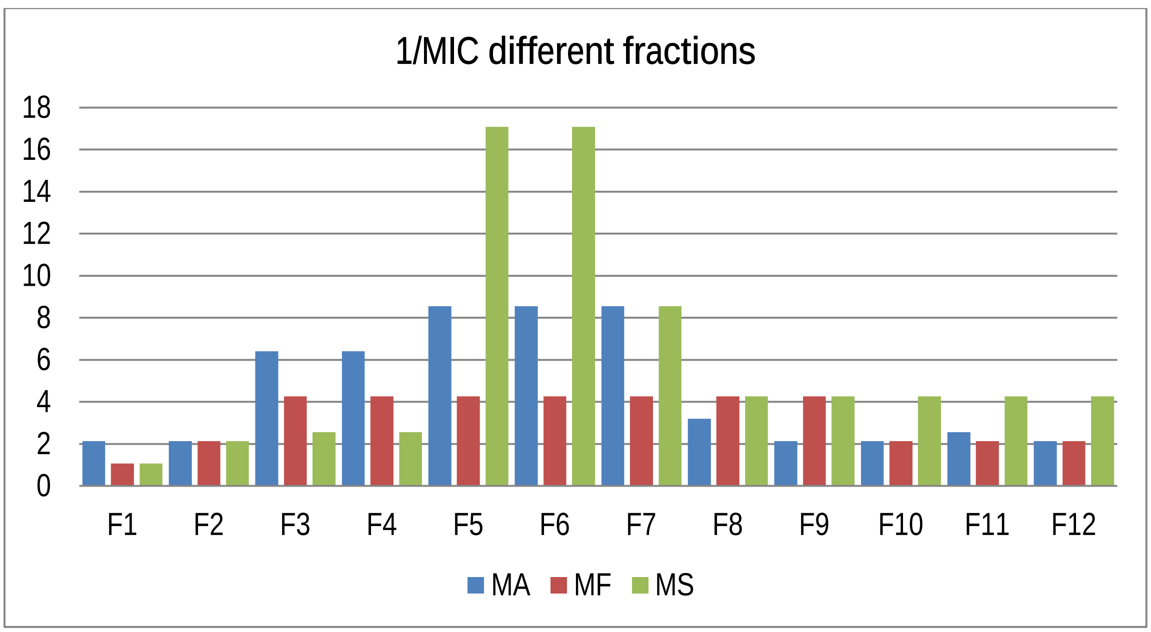


Figure 5.21: The 1/MIC values (mg/mL) of each fraction against the different mycobacteria species. MA: *M. aurum*, MF: *M. fortuitum*, MS: *M. smegmatis*

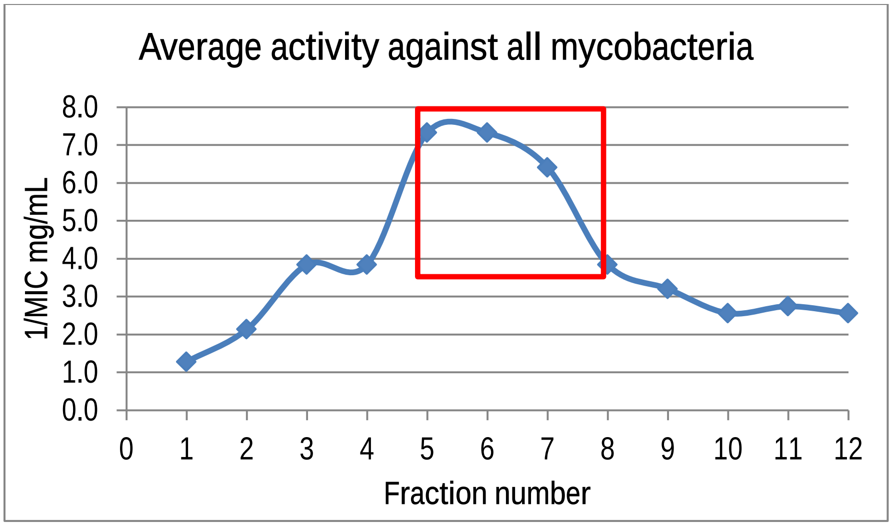


Figure 5.22: The average 1/MIC values (mg/mL) of each fraction against all the different mycobacteria species. Fractions F5, F6 and F7 show consistent high activity

The total activity value of a fraction indicates which species could be candidates for organic production of active extracts for use by communities because it takes into account not only the MIC of the extract but also the yield of the fraction. By dividing the extraction yield in mg/g by the MIC in mg/mL the total activity is calculated in mL/g (Eloff, 2000) as represented in Table 5.4. Among all 12 fractions, F3 had the highest total activity on its own of 19 765 mL/g for *M aurum* followed by 13 162 mL/g for *M. fortuitum* and 7 896 mL/g for *M. smegmatis.* F3 also had active compounds and good MIC values but was not selected at this initial stage as it did not have a consistent similar pattern of phytochemical active compounds on bioautography when compared to F5 to F7 (Figures 5.14-5.18). In future investigations, the isolation of compounds focusing only on fraction F3 will be undertaken. The next fraction showing the highest activity was F6 with a total activity of 19 793 mL/g against *M smegmatis* followed by 9 897 mL/g for *M. aurum* and the lowest 4 943 mL/g for *M. fortuitum* then F5 showing the highest total activity of 17154 mL/g against *M. smegmatis*, followed by 8 577 mL/g for *M. aurum* and 4 284 mL/g for *M. fortuitum* then the last fraction with lower total activity F7 showing 7 262 mL/g for both *M. aurum* and *M. smegmatis* and 3 627 mL/g for *M. fortuitum* (Figure 5.24). The average total activity of the combined fractions was 27 599 mL/g which is higher than F3 alone at 19 765 mL/g (Figure 5.23). At this preliminary stage, this determined the focus on the combination of these three fractions based on their MIC values against all the mycobacterial species.

Table 5.4 The MIC values and total activity of each fraction

Fractions	Mass (mg)	MIC (mg/mL)			Total activity (mL/g)			
		MA Ave	MF Ave	MS Ave	MA	MF	MS	average
F1	2 309	0.47	0.94	0.94	4 926	2 463	2 463	3 284
F2	509	0.47	0.47	0.47	1,086	1 086	1 086	1 086
F3	3 083.3	0.16	0.23	0.39	19 765	13 162	7 896	13 608
F4	1 122.9	0.16	0.23	0.39	7 198	4 794	2 876	4 956
F5	1 003.5	0.12	0.23	0.06	8 577	4 284	17 154	10 005
F6	1 157.9	0.12	0.23	0.065	9 897	4 943	19 793	11 544
F7	849.6	0.12	0.23	0.12	7 262	3 627	7 262	6 050
F8	1 980.8	0.31	0.23	0.23	6 339	8 456	8 456	7 750
F9	1 165.7	0.47	0.23	0.23	2 487	4 976	4 976	4 146
F10	1 768.6	0.47	0.47	0.23	3 773	3 773	7 550	5 032
F11	858.5	0.39	0.47	0.23	2 198	1 831	3 665	2 565
F12	557	0.47	0.47	0.23	1 188	1 188	2 378	1 585

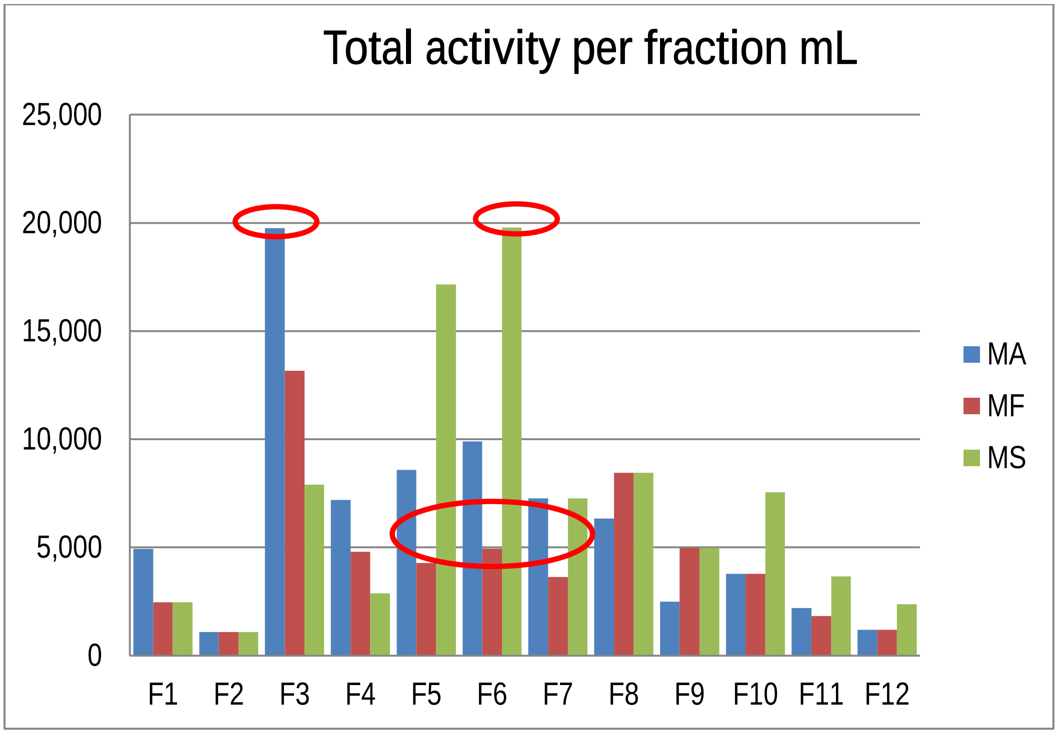


Figure 5.23: Total activity of different fractions (mL/g); Fractions F3 and F6 had the highest total activity for *M. aurum* and *M. smegmatis* respectively followed by fractions F5, F6 and F7 that had better total activity against all the mycobacteria which also correlated with the intensity of active fractions indicated by the clear zone of inhibition on bioautograms

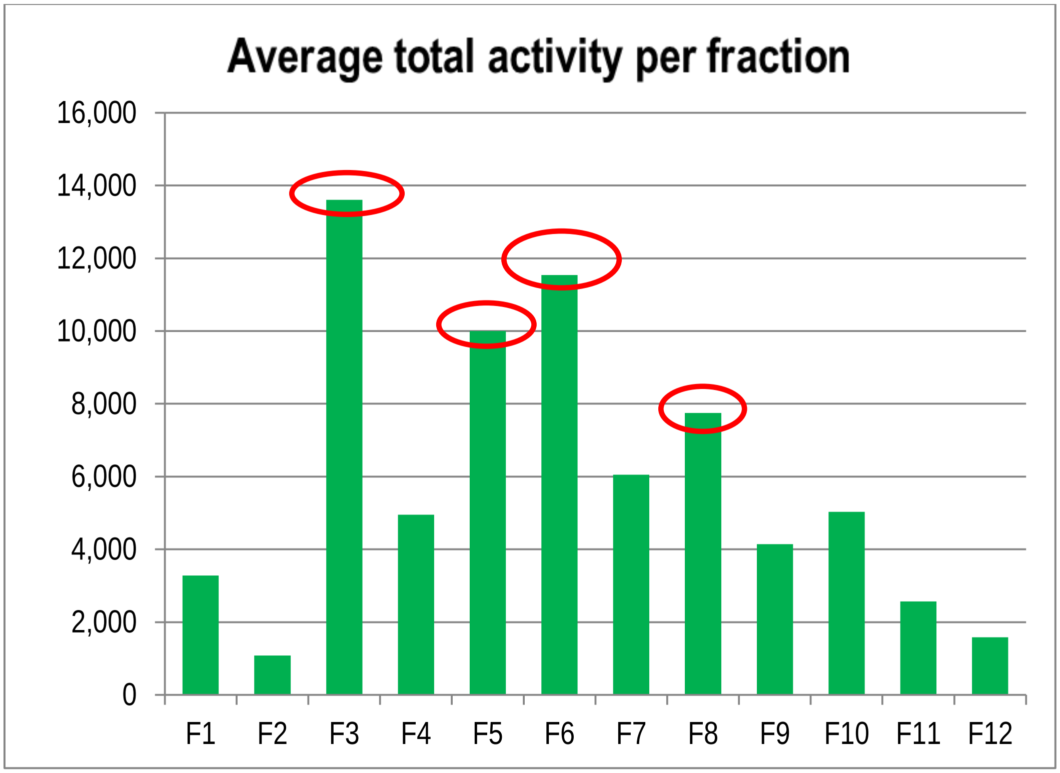


Figure 5.24: The average total activity (mL/g) per fraction

The chloroform fraction revealed the presence of several potential active fractions on the bioautogram sprayed with *M. aurum* whereas on the one sprayed with *M. fortuitum* there were fewer active fractions. This is explained by the different sensitivity of mycobacteria species towards the potential antimycobacterial phytochemical present in the fractions. The R_f value, also called retardation factor, was used as a fingerprint to trace and identify targeted fractions that had antimycobacterial activity at different stages of the fractionation process. R_f values of the chloroform fraction were calculated by dividing the distance travelled by either the extract or fraction or compound spotted on the vanillin sprayed TLC plate or bioautogram eluted in a specific mobile phase solvent system by the distance travelled by the solvent front. R_f values were calculated for the plates eluted in BEA and sprayed with *M. aurum* or *M. fortuitum* as they were good indicators of antimycobacterial activity in general (Figures 5.9-5.11). In Table 5.5, the different values showed the closeness of some of the active fractions such as R_f = 0.21, 0.26 and 0.34 and 0.36. Different fractions that have travelled the same distance could have the same R_f values, namely fractions with R_f values 0.34. The same applied to the different fractions F1 to F12 that had the same R_f values.

Table 5.5 R_f values of different phytochemical bands observed on the chloroform fraction in BEA

	M. aurum	*M. fortuitum*
Rf values		
1	0.05	0.34
2	0.17	0.45
3	0.21	0.52
4	0.26	0.38
5	0.34	
6	0.36	
7	0.47	
8	0.52	
9	0.61	

5.3.2.2 Minimum inhibitory concentration of compounds

The antimycobacterial activity of the five compounds initially isolated yielded a variety of results. Table 5.6 shows the MIC values of *S. undulata* compounds against different organisms. Each value is the mean of three replications measured in µg/mL (test samples of compounds were prepared at an initial concentration of 1 mg/mL). It is worth noting that some of the compounds formed a jelly like substance when inoculated in the growth medium. Therefore, the growth medium was diluted with ethyl acetate or DMSO at different ratios and organisms' growth was tested prior to carrying out the process. All 5 compounds showed activity against rapidly growing mycobacteria with MIC values ranging from 23.44 to 250 µg/mL. The first compounds 1 and 2 showed activity with the highest MIC values varying from 62.5 to > 250 µg/mL. The MIC values were 62.5 µg/mL for *M. aurum* and *M. fortuitum* and 125 µg/mL for *M. smegmatis* whereas C2 showed the highest MIC value >250 µg/mL against *M. fortuitum* and *M. smegmatis*. Compound 3 (SLN1) had the lowest MIC value of 23.44 µg/mL for *M. fortuitum*; 31.25 µg/mL for *M. aurum* and *M. smegmatis*. Compound PKB and compound 5 (C5 or PKC) both showed activity against all three mycobacteria with the highest MIC of 46.88 µg/mL for C4 against *M. smegmatis*.

Table 5.6 MIC values of compounds isolated from *Searsia undulata*

	MIC (µg/mL) and *Mycobacterium* species tested		
Compounds (µg/mL)	*M. aurum*	*M. fortuitum*	*M. smegmatis*
C1	62.5	125	125
C2	62.5	>250	>250
C3-SLN1	31.25	23.44	31.25
C4-PKB	31.25	31.25	46.88
C5-PKC	31.25	31.25	31.25

5.3.3 Cytotoxicity

5.3.3.1 Crude extracts

Reported in Chapter 4, under section 4.3.4

5.3.3.2 Compounds

Only three compounds were evaluated at the time as there were in sufficient purified quantity for further testing and structure elucidation - all three compounds had low cytotoxicity with CP2 being the least toxic 210 µg/mL (Table 5.7). The 50% lethal concentration (LC_{50}) of the compound was determined by calculating the concentration of compound that will keep half the cells viable relative to the untreated control. For each compound a duplicate assay was conducted (Table 5.7). For the first compound 1 (C1), it was found that Y was 0.955 and 0.997 and LC_{50} 27.50 and 35.23 µg/mL for assays 1 and 2, respectively; for C2, Y was the same as for C1 with LC_{50} 172.74 and 248.33 µg/mL for assays 1 and 2 and C3 LC_{50} 48.59 and 46.94 for each of the assays.

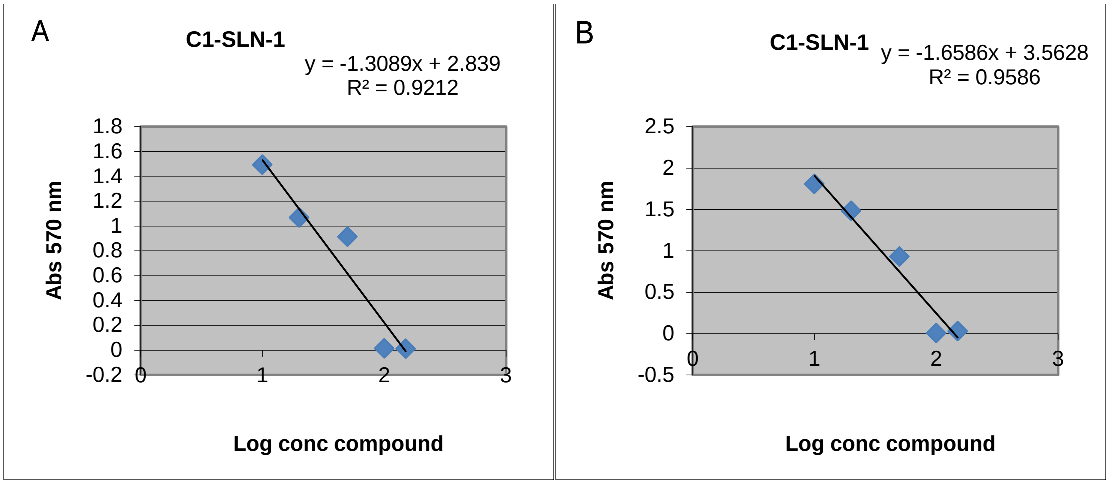


Figure 5.25: LC_{50} of compound 1 where Y was 0.955 (A) and 0.997 (B)

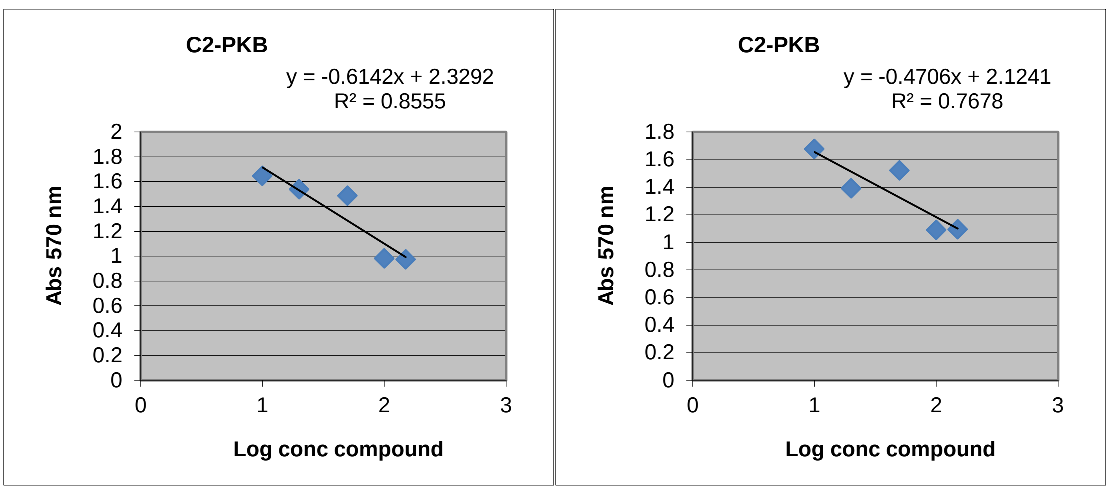


Figure 5.26: LC_{50} of compound PKB where Y was 0.955 (A) and 0.997 (B)

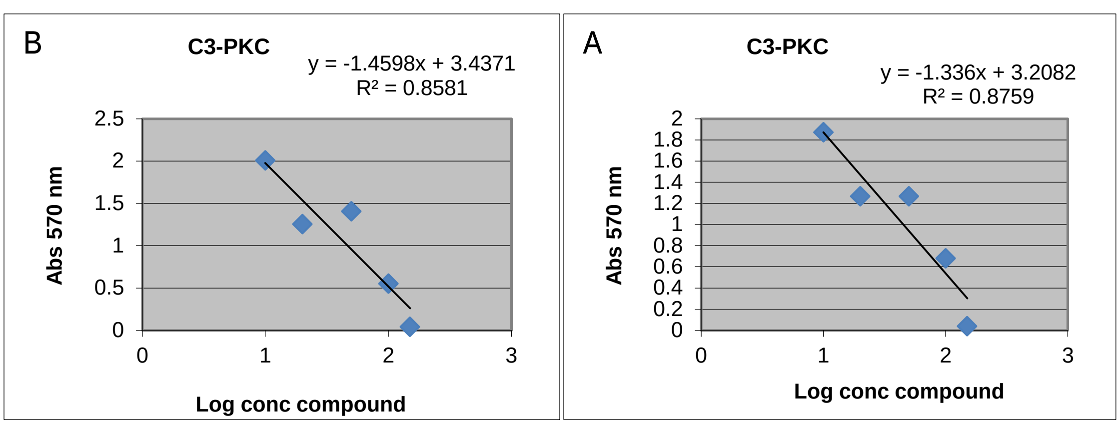


Figure 5.27: LC_{50} of compound 1 where Y was 0.955 (A) and 0.997 (B)

Table 5.7 Cytotoxicity of compounds on Vero Monkey Kidney cells

	Assay 1	Assay 2	Average	SD
Compound 1 (µg/mL)	27.50	35.23	31.37	5.47
Compound 2 (µg/mL)	172.74	248.33	210.53	53.45
Compound 3 (µg/mL)	48.60	46.94	47.76	1.16

It is worth noting that the highest concentration of compound tested was 200 µg/mL and compound 2 was the least toxic out of the three compounds (Table 5.7).

5.3.4 Structure elucidation and identification of compounds

5.3.4.1 SLN1

The initial inspection of the proton spectrum of the SLN1 compound revealed that it was triterpenoid in nature, based on the presence of at least six methyl groups in the spectrum. The compound was approximately 70% pure. Additionally, a pair of triplet-like signals at δ 4.72 ppm and δ 4.59 ppm, integrating to a single proton each, pointed to the presence of a terminal methylene system, likely a 1, 1-disubstituted alkene typical of the lupane pentacyclic triterpenoids. The absence of one of the seven methyl signals typically seen in the lupine systems indicated that one of these had been oxidized or modified, typical of the betulin-type lupane triterpenoids. Direct integration of the material was complicated by the presence of approximately 30% of another compound(s), and the exact number of protons could not be deduced directly from this spectrum.

Inspection of the carbon spectrum indicated the presence of a keto moiety (δ 216.57 ppm), a carboxylate (δ 177.65 ppm) and a typical terminal 1,1-disubstituted alkene (δ 151.70 ppm and δ 110.10 ppm; cf. δ 146 ppm and δ 117 ppm typical of an internal alkene). The absence of signals between δ 65 – 90 ppm indicated that no further oxygenation was present in the system, indicating three oxygen atoms as part of the chemical formula only. Taking into account the purity of the material, 30 distinct carbon signals could be deduced from these data, indicating a chemical formula of $C_{30}H_{46}O_3$.

The initial library searches of the selected carbon signals within Structure Elucidator indicated that sections of the typical lupane system were present, with a 3-keto group, 17-COOH group and 19-propylene group likely, as suspected.

gHSQC_PS in multiplicity-edited form allowed unambiguous assignment of the multiplicities (C, CH, CH_2 or CH_3) of each signal, allowing direct determination of the integration needed in the proton spectrum. Based on the above, it was deduced that there were six methyl groups, eleven methylene groups, five methine groups and eight quaternary carbons, indicating a chemical formula of $C_{30}H_{45}O_3$ but the presence of a carboxylate indicates that the formula should be $C_{30}H_{46}O_3$, to account for the exchangeable –OH proton of the carboxylate.

gDQCOSY (showing direct proton-proton scalar coupling in most cases) showed a clear cross-correlation between the methylene protons at δ 4.72 ppm and δ 4.59 ppm and a broad signal at δ 1.70 ppm corresponding to a methyl group, typical of the interaction seen between the protons on C29 and the methyl group of C30 in a lupane system. Other COSY scalar couplings are indicated in the diagram (Figure 5.28). The above findings are summarized in Table 5.8.

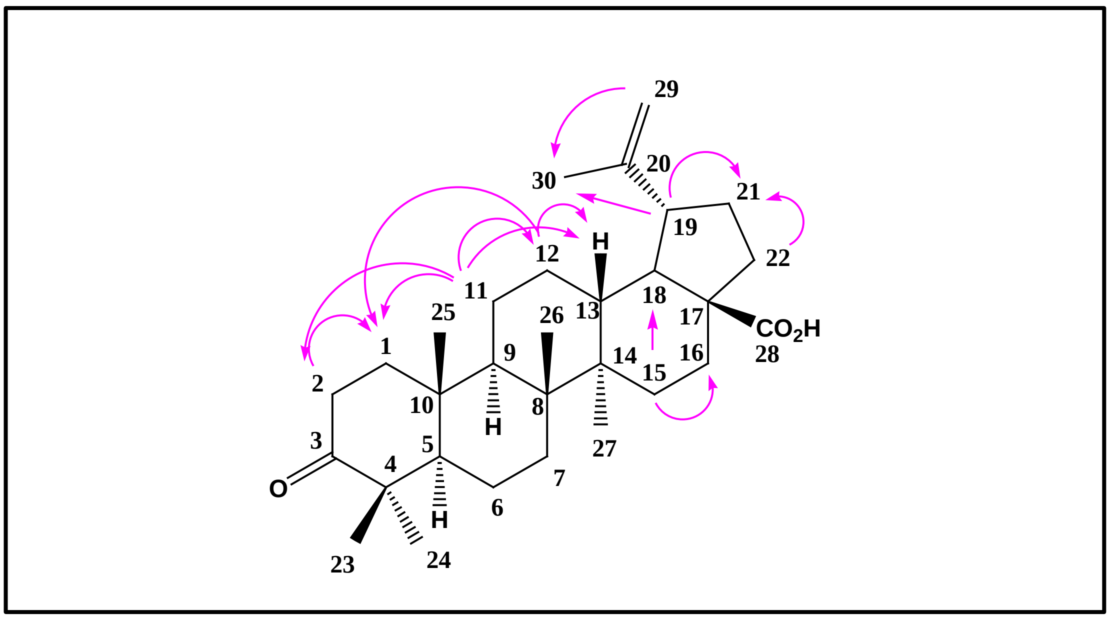


Figure 5.28: Interactions between protons on C29 and methyl group of C30 and other COSY scalar couplings

Due to serious signal overlap in the sub-3 ppm area, many of the correlations could not be distinguished, accounting for the seeming dearth of correlations for the saturated alkane regions of some parts of the molecule. gHMBC correlation spectroscopy enabled direct correlation of protons to carbon atoms three or more bonds away. In this way, the following correlation "map" could be built from the data:

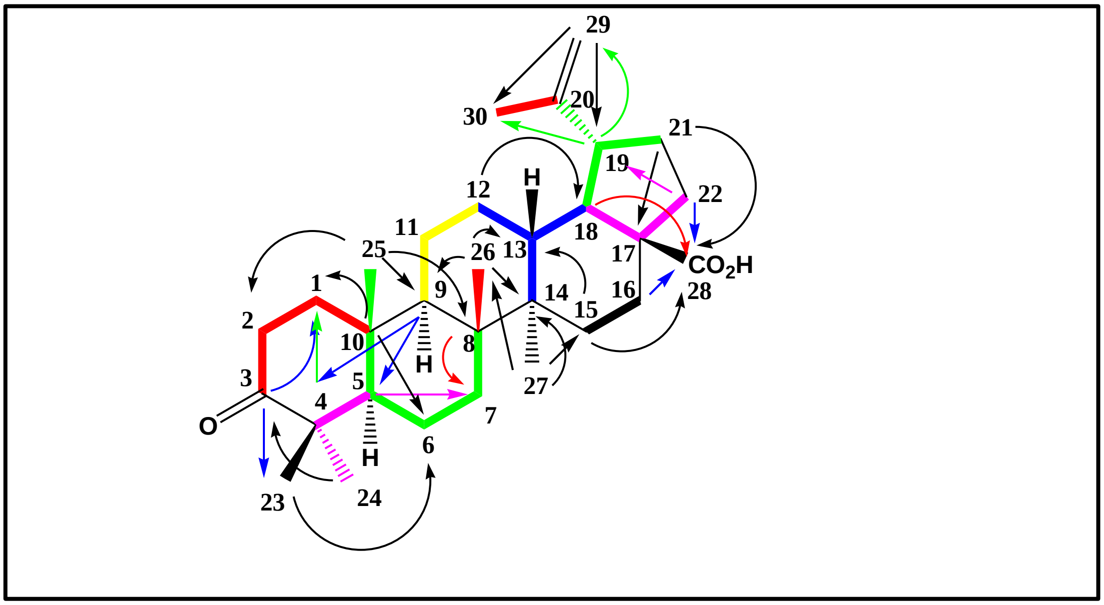


Figure 5.29: Atomic interactions within a network

Each colour in Figure 5.29 refers to a specific atom interacting in a network (shown as either solid bonds or arrows). Significantly, the presence of the exocyclic propylene system C20/C29/C30 is clearly shown by correlation to C19, while the carboxylate C28 is established as attached to C17 indirectly by correlations from H15, H16, H18, H21 and H22. Similarly, the keto moiety at C3 is unambiguously shown from correlations to H1, H2, H23 and H24. The remaining systems confirm the lupane skeleton. Compound SLN1 was identified as betulonic acid with formula $C_{30}H_{46}O_3$ (Figure 5.30).

Table 5.8 [1]H and [13]C NMR of SLN1 (betulonic acid) showing the chemical shift similarities with those obtained by Letedi *et al*. (2014)

C	C mult (HSQC)	δ_x / ppm	H	δ_H / ppm	H mult (*J* / Hz)	gDQCOSY	gHMBCAD
1	CH_2	40.311	1a	1.44-1.47	m	-	-
			1b	1.86-1.93	m	-	
2	CH_2	34.599	2a	2.34-2.42	m	H1b	C1, C10
			2b	2.458	ddd (16.52, 9.78, 7.15)	H1a, H1b	C1, C3
3	C	216.665	-	-	-	-	H1b, H23
4	C	48.037	-	-	-	-	H1b, H29a, H29b
5	CH	55.608	5	1.36-1.45	m	-	C4, C7, C24
6	CH_2	20.459	6a	1.45-1.50	m	-	C1, C5, C8, C10, C25
			6b	1.530	d (1.81)	H6a	C7
7	CH_2	34.541	7	1.44-1.48	m	-	C7
8	C	41.563	-	-	-	-	H7, H26
9	CH	50.006	9	1.661	t (11.18)	H29a	C4, C5
10	C	37.755	-	-	-	-	H1b, H6b
11	CH_2	22.341	11a	1.27-1.33	m	H11b, H12b	C9, C12
			11b	1.44-1.47	m	H1b, H2a, H13	
12	CH_2	26.537	12a	1.06-1.14	m	H11b, H12b, H13	C18
			12b	1.72-1.76	m	H1a, H11a, H12a	
13	CH	39.257	13	2.34-2.42	m	H12a, H12b	C12, C14, C18
14	C	43.394	-	-	-	-	H26, H27
15	CH_2	30.557	15a	1.206	dt (13.42, 3.11)	H15b	C13, C16, C28
			15b	1.558		H15a, H16a, H18	C16
16	CH_2	32.871	16a	2.249	dt (12.80, 3.30)	H15b, H16b	C15
			16b	1.466		H16a	C15
17	C	56.882	-	-	-	-	H21a
18	CH	50.761	18	1.42-1.55	m	-	C28
19	CH	47.788	19	3.049	br td (11.00, 5.22)	H21a, H30	C13, C18, C20, C21, C29, C30
20	C	151.698	-	-	-	-	H30
21	CH_2	31.450	21a	1.36-1.41	m	H19, H21b	C17
			21b	1.90-1.95	m	H15a	C28
22	CH_2	37.624	22a	1.42-1.55	m	H22b	C17
			22b	1.90-1.95	m	H21a, H22a	C18, C19
23	CH_3	27.086	23	1.034	s	-	C6
24	CH_3	21.396	24	0.992	s	-	C3, C4, C5
25	CH_3	16.380	25	0.997	s	-	C2, C8. C9
26	CH_3	16.446	26	0.950	s	-	C9, C13, C26

27	CH_3	15.040	27	1.035	s	-	C15, C20, C24, C26, C7, C29, C30
28	C	177.65	-	-	-	-	H16b, H22b
29	CH_2	110.097	29a	4.592	br t (1.70)	H29b, H30	C19, C30
			29b	4.724	br d (2.20)	H29a	C19, C30
30	CH_3	19.595	30	1.701	br s	H13, H29b	C19, C20, C29

d = doublet, ddd = doublet of doublets of doublets, dt = doublet of triplets, br = broad, t = triplet, td = triplet of doublets, s = singlet, m = multiplet, mult = multiplicity, *J* = scalar coupling constant in Hertz, δ_X = chemical shift of nucleus X in ppm.

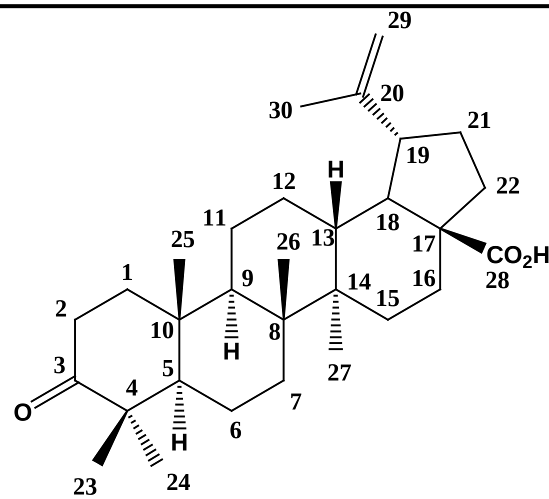


Figure 5.30: The structure of betulonic acid (SLN1)

5.3.4.1.1 Physico-chemical properties and reported biological activities of betulonic acid and derivatives

Betulonic acid can be obtained by oxidation of the following compounds: betulin, betulinic acid, betulonal or betulinal, but generally as starting material betulin is used, mainly because is highly accessible as a naturally-occurring compound (Bastos *et al*., 2007; Tolstikov *et al*., 2005). During this study, betulonic acid [lup-20(29)-en-3-oxo-28-oic] was obtained as a yellow powder, highly non-polar with a formula of $C_{30}H_{46}O_3$ (Ledeţi *et al*., 2015; Melnikova *et al*., 2012). Until the 2000s, interest in betulinic acid was primarily due to its role as the precursor for synthesis of betulonic acid, which is an effective drug against human melanoma and as an anti-inflammatory and anti-viral (Bastos *et al*., 2007; Laavola *et al*., 2016; Melnikova *et al*., 2012; Moldovan *et al*., 2013; Periasamy *et al*., 2014). According to ChemFaces material safety data sheet (2015), betulonic acid amide isolated from the branch of *Eucalyptus globulus* Labill. stimulates the regenerative response in hepatocytes under conditions of combined toxic exposure characterized by a significant decrease of the degree of severity of liver fibrosis and the absence of cirrhotic transformation of the liver. It was also observed that betulonic acid derivatives have promising cytostatic activity *in vitro* and could be used as potential leads for the development of new type of anti-cancer agents (Bastos *et al*., 2007; Gheorgheosu *et al*., 2014).

The mechanism of antiproliferative action of betulonic acid is thought to occur via induction of apoptosis through the mitochondrial intrinsic pathway. Other biological properties are anticholestatic effects in mice, antiviral, anti-inflammatory, antimicrobial, hepatoprotective and immunostimulant activities (Dehelean *et al*., 2012; Flekhter *et al*., 2000; Melnikova *et al*., 2012; Periasamy *et al*., 2014; Tolstikov *et al*., 2005; Yang *et al*., 2015).

Betulonic acid can be synthesized; it can be obtained by oxidation of betulin by Jones' reagent made of Cr(VI) compounds in aqueous acetone (CrO_3/H_2SO_4/acetone) on solid supports such as alumina, zeolites and silica gel at room temperature. Selective oxidation (100%) during 30 min of betulin up to betulonic aldehyde was determined when silica gel support was used. It was found that the selective oxidation to betulonic acid is due to the influence of Al^{3+} ions. The synthesis of betulonic acid is long, not very profitable and requires challenging purification steps including column chromatography, multiple recrystallizations and extraction using very large volumes of solvents, making it unsuitable for extensive scale industrial application (Bastos *et al*., 2007; Tolstikova *et al*., 2006).

Betulinic and betulonic acid are naturally occurring triterpenes found in many plants but betulonic acid can be obtained from oxidation of betulinic acid with Jones' reagent. Betulonic acid is a derivative of betulinic acid. Betulinic acid is a naturally occurring pentacyclic triterpenoid which has antiretroviral, antimalarial and anti-inflammatory properties as well as anticancer activity. It is found in the bark of several species of plants, principally the white birch (*Betula pubescens*) from which it gets its name, but also from the ber tree (*Ziziphus mauritiana*), selfheal (*Prunella vulgaris*), the tropical carnivorous plants *Triphyophyllum peltatum* and *Ancistrocladus heyneanus, Diospyros leucomelas*, jambul (*Syzygium formosanum*), flowering quince (*Pseudocydonia sinensis*, formerly *Chaenomeles sinensis* (koehne), rosemary and *Pulsatilla chinensis*. The pure compound of betulinic acid (oxidation product of betulin) appears as a white crystalline solid, melting at 295-297°C. It was isolated from *Melaleuca cajuput* and was chromatographed on a silica gel column using chloroform as eluent (Abd Rahman *et al*., 2012; Bastos *et al*., 2007; Cragg and Newman, 2005; Faujan *et al*., 2010; Mahajan and

Chopda, 2009; Newman and Cragg, 2007). It exhibits limited solubility in organic alcohols such as methanol and ethanol, chloroform and ether and has low solubility in water, petroleum ether, dimethylformamide, dimethyl sulfoxide, and benzene. It is highly soluble in pyridine and acetic acid (Cichewicz and Kouzi, 2004). It is not readily visible on thin layer chromatography plates under UV (254 and 365 nm) (Cheng *et al*., 2011; Jäger *et al*., 2007) but it is easily detected following exposure to iodine vapors, anisaldehyde-sulphuric acid, or vanillin (Periasamy *et al*., 2014).

5.3.4.2 Compound-PKB

The sample was analysed by NMR spectroscopy and high resolution mass spectrometry. The proposed structure is given in Figure 5.30. The structure was assigned on the basis of 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC and HMBC) data. The NMR data was in agreement with the proposed structure (Table 8). The molecular formula was deduced as $C_{30}H_{18}O_{10}$ on the basis of its positive MS data which showed the presence of an $[M+H]^+$ ion at m/z 539.0959 as indicated in Figure 5.31.

This compound could possibly be new as no matching structure was found from Scifinder search. A second plant material collection will be planned to extract a larger amount of the compound for further analysis which will include crystallization, melting point and Fourier Transform Infrared Spectroscopy (FTIR).

Table 5.9: 1 D and 2 D NMR data of compound PK-B in DMSO-d6

^{1}H	COSY	HSQC	HMBC[a]
13.30 (1H, brs, OH-5)	—	—	60.0 (C, C-5), 109.2 (C, C-4a), 104.1 (CH, C-6)
12.70 (1H, s, OH-5'')	—	—	161.3 (C, C-5''), 101.9 (C, C-4''a), 99.3 (C, C-6'')
10.63 (2H, brs, 2 X OH)	—	—	—
9.69 (1H, brs, OH)	—	—	—
7.67 (2H, d, *J* = 8.8 Hz, H-2' & H-6')	6.82 (H-3' & H-5')	128.6 (2 X CH, C-2' & C-6')	165.0 (C, C-2), 161.6 (C, C-4'), 128.6 (2 X CH, C-2' & C-6')
7.32 (2H, d, *J* = 8.6 Hz, H-3''' & H-5''')	6.87 (H-2''' & H-6''')	131.7 (2 X CH, C-3''' & C-5''')	157.3 (C, C1'''), 131.7 (2 X CH, C-3''' & C-5'''), 114.3 (C, C-8)
7.04 (1H, s, H-3)	—	103.3 (CH, C-3)	183.0[b] (C, C-4), 165.0 (C, C-2), 120.8 (C, C-1'), 109.2 (C, C-4a)
7.02 (1H, s, H-6)	—	104.1 (CH, C-6)	160.0 (C, C-5), 152.8 (C, C-7), 114.3 (C, C-8), 109.2 (C, C-4a)
6.87 (2H, d, *J* = 8.6 Hz, H-2''' & H-6''')	7.32 (H-3''' & H-5''')	115.1 (2 X CH, C-2''' & C-6''')	157.4 (C, C1'''), 119.8 (C, C4''')
6.82 (2H, d, *J* = 8.8 Hz, H-3' & H-5')	7.67 (H-2' & H-6')	115.9 (2 X CH, C-3' & C-5')	161.6 (C, C-4'), 120.8 (C, C-1')
6.31 (1H, d, *J* = 2.0, H-8'')	6.19 (H-6'')	93.9 (CH, C-8'')	164.2 (C, C-7''), 154.8 (C, C-8''a), (101.9, C-4'' a), 99.3 (CH, C-6'')
6.19 (1H, d, *J* = 2.0, H-6'')	6.31 (H-8'')	99.3 (CH, H-6'')	164.2 (C, C-7''), 161.3 (C, C-5''), 101.9 (C, C4''a), 93.9 (CH, C-8'')
5.45 (1H, s, H-3'')	—	88.8 (CH, H-3'')	182.8[b], 165.5, 101.9 (C, C4''a)

a HMBC correlations not observed for C-8a at σ_C 154.0

b Assignments may be interchanged

Compound PK-B formula $C_{30}H_{18}O_{10}$

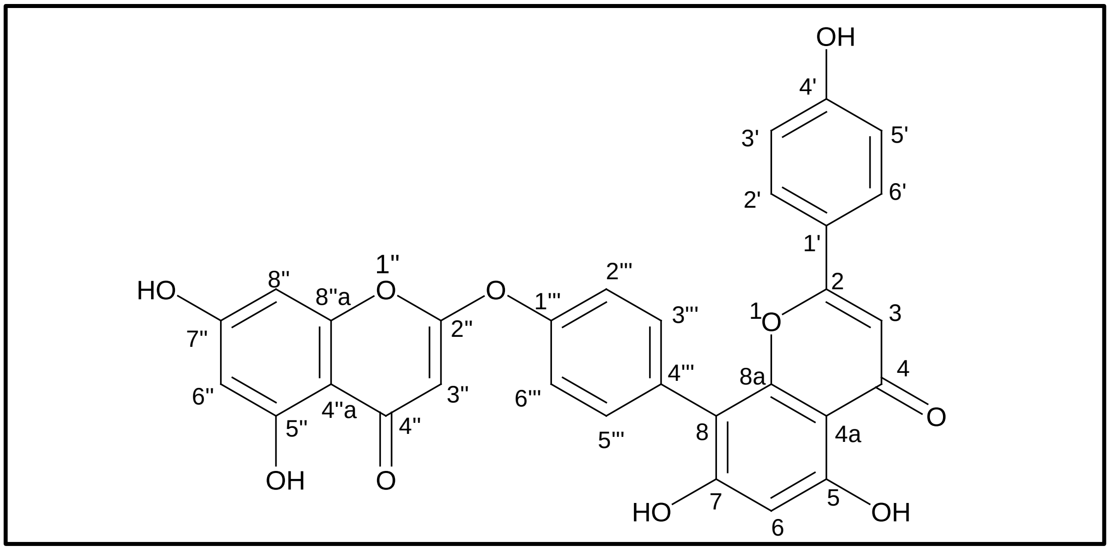


Figure 5.31: Proposed structure for sample PK-B

5.4 CONCLUSION

To our knowledge, this is the first report of the isolation of betulonic acid from the chloroform fraction of *S. undulata*. The NMR data of the isolated betulonic acid was consistent with previous publications (Ledeti *et al*., 2014; Ledeţi *et al*., 2015; Melnikova *et al*., 2012). Its biological properties have been reported, which include antitubercular and antiproliferative (antitumor activity) (Bastos *et al*., 2007; Dehelean *et al*., 2012; Periasamy *et al*., 2014; Yang *et al*., 2015). *Searsia undulata* was found to be toxic to C3A (Human cancer liver cell) but less toxic to normal Vero kidney cells and this potential anticancer activity should be further investigated.

CHAPTER 6:
SUMMARY AND CONCLUSIONS

This study comprised two phases: phase I comprised the isolation and characterization of *Mycobacterium* spp. During phase II, extracts of selected plant species of the family Anacardiaceae were tested for antimycobacterial activity using some isolates from phase I and strains of non-tuberculous mycobacteria. Several objectives were formulated and reported results obtained from these objectives are summarized in this chapter:

6.1 ISOLATION AND CHARACTERIZATION OF *MYCOBACTERIUM* SPECIES

This objective was achieved through the processing of samples, isolation, identification, characterization and antimycobacterial activity of some of the isolates.

The samples from slaughtered animals showing lesions suggestive of tuberculosis and cattle positive reactors to tuberculin test were processed. Samples received included five different animal species that included black wildebeest (*Connochaetes gnou*), cattle (*Bos taurus*), impala (*Aepyceros melampus*), rabbit (*Oryctolagus cuniculus*) and warthog (*Phacochoerus africanus*). Methods used were isolation in culture using liquid medium BACTEC^{TM} MGIT^{TM} 960, and solid growth media on Löwenstein Jensen slants with glycerol and pyruvate. The isolates were further identified using the commercial kit GenoType CM/AS reverse line blot assay and DNA strip *Mycobacterium* identification species (Hain Life Science, Gmbh Nehren, Germany), biochemical substrate, characterized by multiplex PCR, spoligotyping, variable number of tandem repeat (VNTR), gene sequencing, phylogenetic analysis and antimicrobial susceptibility test of cattle isolates (*M. bovis*) against first-line TB drugs using Genotype® MTBDR*plus* kit (Hain Life Science Gmbh Nehren, Germany).

Samples from cattle yielded 15 isolates of *M. bovis* of which 53% were resistant to the first-line drugs INH and rifampin (MDR-*M. bovis*)-these findings are of concern as there are very few data available in South Africa related to human tuberculosis caused by *M. bovis*. Considering that South Africa is among the list of high burden MDR/XDR-TB countries, accurate diagnostic and antimicrobial sensitivity patterns are necessary for the implementation of effective treatment regimens. Further investigation on the prevalence and incidence of *M. bovis* in humans and characterization of such isolates is needed.

The isolates from cattle were further analysed by spoligotyping and yielded two spoligotypes. SB0121 (67%) and SB 1235 (33%). The VNTR was type 1. These isolates were from the same origin. Spoligotype are interesting epidemiological tools to trace the origin of the strain for the control/management of the disease.

Samples from black wildebeest yielded non-tuberculous mycobacteria: a novel *Mycobacterium Avium* Complex species and *Mycobacterium avium* subsp. *hominissuis*. The sample from a rabbit also yielded *M. avium* subsp*. hominissuis*. These findings were of concern as meat from black wildebeest and rabbit are used for human consumption. Non-tuberculous mycobacteria are among emerging mycobacteria with increased interest as their clinical significance in causing disease has been established in humans but not yet in animals. Adding to that, some of the members of the MAC have been reported as potentially zoonotic which includes *M. avium* subsp. *hominissuis*. Further investigation is needed to establish the pathogenicity of non-tuberculous mycobacteria in animals beyond any doubt and deposition of new isolates at the international gene data bank.

Samples from warthogs yielded a mixture of *M. bovis* and non-tuberculous mycobacteria, namely *M. intracellulare* and *M. avium*. The sample from an impala yielded *M. bovis.*

6.2 ANTIMYCOBACTERIAL ACTIVITY OF ACETONE LEAF EXTRACTS OF PLANT SPECIES FROM ANACARDIACEAE FAMILY

This objective was achieved by selecting 15 plant species from the Anacardiaceae family and conducting *in vitro* evaluation of antimycobacterial and cytotoxicity activities of crude acetone extracts followed by selection of the most active extract for bioassay-guided isolation of active antimycobacterial compounds. Fifteen plant species were screened (*Harpephyllum caffrum, Heeria argentea, Lannea discolor, Loxostylis alata,* *Ozoroa mucronata, Ozoroa paniculosa, Protorhus longifolia, Searsia chirendensis, Searsia lancea, Searsia leptodictya, Searsia magaliesmontana* subsp*., Searsia pyroides, Searsia undulata, Sclerocarya birrea* subsp *caffra* and *Smodingium argutum*). The acetone extraction yield of the plant species ranged from 0.8 to 18.8%. The highest percentage was observed with *Searsia magaliesmontana* (18.8%) followed by *Searsia undulata* (12.5%) and *Protorhus longifolia* (8.3%). *Heeria argentea* had the lowest yield of 0.8%.

The antimycobacterial activity of acetone leaf extracts of each plant species was determined using a twofold serial dilution method against pathogenic (*M. bovis*, MDR-TB), ATCC *M. tuberculosis H37Ra* and rapidly growing mycobacteria that included ATCC and NCTC cultures, *M. aurum*, *M. fortuitum* and *M. smegmatis.*

The number of antimycobacterial compounds against rapidly growing mycobacteria in each extract was determined using bioautography. The plates were developed in duplicate, one for bioautography and the other plate for visualization of phytochemicals present in each plant following spraying with the chromogenic reagent vanillin-sulphuric acid. Bioautography of extracts using two *(M. aurum* and *M. fortuitum)* out of the three rapidly growing mycobacteria led to strong, clear zones of inhibition. This also corresponded with low MIC values obtained, ranging from 50 to 100 µg/mL.

All 15 plant species had good to moderate antimycobacterial activity against rapidly growing and pathogenic mycobacteria ranging from 50 to 590 µg/mL. This confirms the results obtained against *M. smegmatis* in the Phytomedicine Programme database. The following five plant species MIC values were good and showed the presence of antimycobacterial compounds on bioautograms: *Searsia undulata* extracts had significant antimycobacterial activity with the lowest MIC value of 70 µg/mL for *M. aurum* and *M. fortuitum* followed by *M. smegmatis* with MIC of 90 µg/mL. *Protorhus longifolia* extracts also had significant antimycobacterial activity against all three non-tuberculous mycobacteria with MIC values of 110 µg/mL for both *M. aurum* and *M. fortuitum* and 70 µg/mL for *M. smegmatis*. *Searsia lancea*, *Sclerocarya birrea* and *Harpehyllum caffrum* had moderate activity of 420 µg/mL, 520 µg/mL and 590 µg/mL against *M. aurum* respectively whereas the same plant species had moderate activity with low MIC values of 210 and 110 µg/mL; 130 and 210 µg/mL and 210 and 110 µg/mL against *M. fortuitum* and *M. smegmatis,* respectively. *Searsia undulata* extracts had significant activity against all mycobacteria including *M. bovis* and MDR-TB isolates with MIC ranging from 50 to 110 µg/mL, and the best activity with an MIC of 50 µg/mL against *M. tuberculosis* ATCC strain H37Ra. Therefore, this species was selected for further investigation. Positive correlation was observed between the activity of the pathogenic isolates, *M. bovis and MDR-TB,* with *M. fortuitum* with (r) coefficient correlation values of 0.62 and 0.65 respectively whereas negative correlation with value of -0.005 was observed with *M. aurum* and the pathogenic mycobacteria. Good correlations were obtained between the ATCC strain, H37Ra, and rapidly growing mycobacteria with r values of 0.92, 0.87 and 0.37 for *M. fortuitum*, *M. aurum* and *M. smegmatis* respectively and the highest coefficient correlation value of 0.98 between *MDR-TB* isolate with the pathogenic *M. bovis* isolate.

6.2.1 Antimycobacterial activity of fractions and compounds of *S. undulata*

6.2.1.1 Minimum Inhibitory concentration of fractions

The twelve fractions obtained from the chloroform fraction following solvent-solvent fractionation and column chromatography were tested against the three rapidly growing mycobacteria, *M. aurum*, *M. fortuitum* and *M. smegmatis*. All fractions had good antimycobacterial activity with MIC values ranging from 58 to 468 µg/mL which were related in most cases to the presence of clear zones of inhibition on bioautograms.

The fractions F5, F6 and F7 had similar compounds on bioautograms and were combined for further fractionation to isolate antimycobacterial compounds. These fractions had MIC of 117 µg/mL for *M. aurum*, 234 µg/mL for *M. fortuitum* and 58 µg/mL for *M. smegmatis* except F7 that had a MIC of 117 µg/mL for *M. smegmatis.* Fractions F5 and F6 had the best activity with MIC of 58 µg/mL observed against *M. smegmatis*.

6.2.1.2 Minimum inhibitory concentration of isolated compounds

Five compounds were isolated and tested against *M. aurum*, *M. fortuitum* and *M. smegmatis*. All 5 compounds showed activity against rapidly growing mycobacteria with

MIC values ranging from 23.44 to 250 µg/mL. The first compounds 1 and 2 had the highest MIC values varying from 62.5 to >250 µg/mL. The MIC values of 62.5 µg/mL were obtained against *M. aurum* and *M. fortuitum* and 125 µg/mL for *M. smegmatis* whereas C2 showed the highest MIC value >250 µg/mL against *M. fortuitum* and *M. smegmatis*. Compound 3 (C3 or SLN1) had the lowest MIC value of 23.44 µg/mL against *M. fortuitum*; 31.25 µg/mL against *M. aurum* and *M. smegmatis*. Compound C4 (C4 or PKB) and compound 5 (C5 or PKC) both showed activity against all three mycobacteria with best value of 46.88 µg/mL for C4 against *M. smegmatis*.

6.3 CYTOTOXICITY OF ACETONE LEAF EXTRACTS OF PLANT SPECIES FROM ANACARDIACEAE FAMILY

Most drugs or other substances are metabolised in the liver and excreted by the kidney. Mycobacterial species causing tuberculosis multiply and hide in macrophages. This objective was achieved by selecting three cell lines to represent these organs, namely Vero monkey kidney, human liver hepatoma (C3A) and mouse macrophage cells (RAW 264.7). Crude extracts (*Heeria argentea*, *Lannea discolor*, *Protorhus longifolia*, *Searsia undulata* and *Sclerocarya birrea*) with good antimycobacterial activity (from significant to moderate) and those that showed visible zones of inhibition on bioautograms were selected for cytotoxicity testing. The cytotoxicity was determined using a tetrazolium-based colorimetric assay with 3-(4, 5-dimethylthiazol)-2, 5-diphenyl tetrazolium bromide (MTT) as the indicator. All crude extracts tested showed low toxicity against all three cell lines except *Searsia undulata* that had moderate toxicity to C3A cells with LC_{50} of 0.034 mg/mL. This deserves further investigation as a source of anticancer substances as C3A cells are cancer cells with abnormal metabolic activity. The *S. undulata* extract had LC_{50} of 0.50 and 0.12 mg/mL on Vero cells and RAW cells respectively with good selectivity indexes of 7.08 on Vero cells and more than 1 on RAW cells for non-tuberculous and MDR-TB mycobacteria. *Protorhus longifolia* had an LC_{50} of 0.62 mg/mL on C3A cells, 0.88 mg/mL on Vero cells and >1 mg/mL for RAW cells with the highest selectivity index on Vero cells of 12.6 for *M. smegmatis* followed by 8.02 for *M. aurum*, *M. fortuitum* and MDR-TB. Plant extracts with SI values less than 1 mean that the extracts are relatively less toxic to the bacteria and more toxic to the mammalian cells. Therefore, extracts with SI >1 may be relatively safer to use *in vivo* (not accounting for pharmacokinetics parameters) as they are less toxic to mammalian cells but more toxic to the pathogens. The selectivity indexes, especially of *P. longifolia* with SI = 12. 6 could be considered as very promising, as a good therapeutic index for a remedy or drug should be ≥10. It is also worth noting that efficacy "*in vitro*" might differ from efficacy '*in vivo*" due to different parameters influencing pharmacodynamics and pharmacokinetics of drugs administered by different routes to humans and animals. *Protorhus longifolia* and *Searsia undulata* had the highest selectivity index against the three rapidly growing mycobacteria and pathogenic mycobacteria and the highest total activity. These rapidly growing species had the highest total activity of 5 357 mL/g for *S. undulata* and 3 557 mL/g for *P. longifolia* which indicates the volume to which the extract from 1 g can be diluted and still inhibit the growth of mycobacteria. It is also worth noting that the leaves of *S. undulata* are chewed by Khoisan people to treat chest colds, indicating a low toxicity. The mode of administration and interaction with other factors "*in vivo*" must be considered when assessing toxicity. Plant extracts showing sensitivity to cell lines with LC_{50} values >0.1 mg/mL are considered not cytotoxic in terms of searching for anticancer compounds. *Searsia undulata* was selected as the preferred plant species for compound isolation based on its high cytotoxicity on abnormal cells (C3A) (potential candidate for anticancer agent) and low cytotoxicity on the normal Vero kidney cells and its good antimycobacterial activity.

6.3.1 Cytotoxicity of compounds isolated from *Searsia undulata*

The crude extract of *Searsia undulat*a at a concentration of 100 µg/mL had low cytotoxicity (LC_{50} = 500 µg/mL) on Vero cells (Section 6.3). Three of the compounds isolated from *S. undulata* had moderate toxicity with compound 2 (SLN1) being the least toxic (LC_{50} = 211 µg/mL) followed by compound 3 with LC_{50} = 48 µg/mL (PK-B) and compound 1 (LC_{50} = 31 µg/mL). The other two compounds were not tested because they were not pure and not in sufficient quantity.

6.4 ISOLATION OF BIOACTIVE FRACTIONS AND COMPOUNDS FROM THE LEAF OF *SEARSIA UNDULATA*

The five fractions from solvent-solvent fractionation (chloroform, butanol, hexane, H_2O-35% methanol and H_2O) were eluted in BEA solvent system and tested for antimycobacterial activity against *M. aurum*, *M. fortuitum* and *M. smegmatis and MDR-TB* and *H37Ra*. Thin layer chromatography fingerprints eluted in hexane: ethyl acetate at different ratios was used to locate the active phytochemical compounds as mentioned under section 6.2.

The chloroform fraction was selected as the fraction with the best antimycobacterial activity based on good MIC values and presence of more than 10 potential active compounds as discussed under section 6.2.1.1. It was further fractionated using column chromatography packed with silica gel into 55 fractions that were combined into 12 sub-fractions (F1-F12). Three (F5-F7) out of the 12 fractions showing good antimycobacterial activity and presence of active phytochemical compounds on bioautography were combined and were further fractionated using column silica gel chromatography for isolation of compounds.

Among all 12 fractions, F3 had the highest total activity on its own of 19 765 mL/g for *M aurum* followed by 13 162 mL/g for *M. fortuitum* and 7 896 mL/g for *M. smegmatis.* It also had active compounds and good MIC values but was not selected at this initial stage as it did not have the consistent similar pattern of phytochemical active compounds on bioautography and TLC when compared to F5 to F7 (Figures 5.14-5.18). In future investigations, the isolation of compounds from fraction F3 will be undertaken. The next fraction showing the highest activity was F6 with a total activity of 19 793 mL/g against *M smegmatis* then F5 showing the highest total activity of 17154 mL against *M. smegmatis* then the last fraction with lower total activity F7 showing 7 262 mL for both *M. aurum* and *M. smegmatis*. The average total activity of the combined fractions was 27 599 which is higher than F3 alone 19 765 mL. At this preliminary stage, this determined the focus on the combination of these three fractions based on their MIC values against all the mycobacteria species.

The R_f value, or retardation factor, was used to trace and identify targeted compounds with antimycobacterial activity at different stages of the fractionation process. R_f values of the chloroform fraction were calculated by dividing the distance travelled by either the extract or fraction or compound spotted on the vanillin sprayed TLC plate or bioautogram eluted in a specific mobile phase solvent system. R_f values were calculated for the plates eluted in BEA solvent and sprayed with *M. aurum* and *M. fortuitum* as they were good indicators of antimycobacterial activity in general.

Isolation of compounds from the combined semi-purified fractions, F5, F6 and F7 (1019.7mg) was carried out using a small column packed with silica gel with 100% *n*-hexane and fractions collected into honey jars. Compounds were eluted with 100% *n*-hexane with increasing polarity with 2% ethyl acetate resulting in sequential elution of SLN1 (26.2 mg), PKA1 (15.4 mg), PKB (22.4 mg), PKC (15.9 mg) and PKD (8.4 mg). The purified PK B was obtained by using a mixture of chloroform and methanol as eluent on a silica gel column at the second run.

6.5 STRUCTURE ELUCIDATION AND IDENTIFICATION OF COMPOUNDS

6.5.1 Compound SLN1

This compound was collected and dried as a light yellow powder. The initial inspection of the proton spectrum revealed that the material was triterpenoid in nature, based on the presence of at least six methyl groups in the spectrum. The material consisted of about 70% of the major product. Additionally, a pair of triplet-like signals at δ 4.72 ppm and δ 4.59 ppm, integrating to a single proton each, pointed to the presence of a terminal methylene system, likely a 1, 1-disubstituted alkene typical of the lupane pentacyclic triterpenoids. The compound was identified as betulonic acid, which has been reported previously as having antimicrobial activity.

During the process of the isolation of compounds, it is worth noting that the work was carried out at room temperature and samples were exposed to oxygen and light. Changes observed on TLC plates indicated that certain compounds were unstable and decomposed. This could imply that the initial compound was betulinic acid which underwent oxidation to its derivative betulonic acid. In either case, both compounds were reported to have similar biological activity properties.

Betulinic and betulonic acid are naturally occurring triterpenes found in many plants but betulonic acid can be obtained from oxidation of betulinic acid with Jones' reagent.

Betulonic acid is a derivative of betulinic acid as described above. Betulinic acid is a naturally occurring pentacyclic triterpenoid which has antiretroviral, antimalarial and anti-inflammatory properties as well as anticancer (Dehelean *et al*., 2012). This appears to be the first report of isolation of betulonic acid from *S. undulata*.

6.5.2 Compound PK-B

This compound was collected and dried as a slightly yellow powder. Its putative structure did not match any chemical structure on Scifinder. This compound seems to be novel. More of the compound needs to be isolated to enable crystallization, melting point and Fourier Transform Infrared Spectroscopy (FTIR) for full elucidation of the chemical structure. The proposed structure is provided in section 5.3.4.2 (Figure 5.30).

6.6 RESEARCH CHALLENGES

- All bioassays were conducted *in vitro*. There is still a need to validate the efficacy as well as safety *in vivo*. Several parameters and interactions need to be considered during the *in vivo* metabolism of a drug which leads to its effective bioavailability.
- The isolation of compounds was a laborious and difficult process due to the complexity of antimycobacterial active phytochemical compounds that were closely related. This made the separation and purification processes challenging.
- Delay encountered in the appointment of a phytochemist on site for guidance in the isolation of compounds.
- No easy access to NMR instrument and very limited expert staff available therefore the high demand from researchers making use of this technique could not be met within the required time.
- NMR broken probes were sent for repair in Germany (no technical local representation)
- Minimum inhibitory concentration (MIC): the main challenge encountered was the formation of a jelly-like substance by some compounds when inoculated in the growth medium, preventing tested compounds from dissolving. Therefore, the growth medium was diluted with solvents such as ethyl acetate or DMSO at different ratios and organisms' growth was tested prior to carrying out the

bioactivity assay. Bioautography: the use of tested mycobacteria was only limited to the rapidly growing organisms and not extended to the pathogenic mycobacteria. This is due to the technique as a mycobacterial suspension is sprayed on the TLC plates. Pathogenic mycobacteria are highly infectious and air borne infection is one of the main routes of contamination/infection. The slow-growing mycobacteria would also not be able to survive on the TLC plate for the extended period of time necessary for adequate growth.

6.7 GENERAL CONCLUSION, RECOMMENDATIONS AND FUTURE PERSPECTIVES

6.7.1 Isolation and characterization of mycobacterial species

The involvement of some zoonotic *Mycobacterium* spp. in the disease-causing process has been established. There is evidence that new strains of bacteria are circulating. It has also been reported that clinical signs of disease caused by *M. bovis* and *M. tuberculosis* are indistinguishable. Other findings mention that radiological clinical pictures of some pulmonary diseases caused by non-tuberculous mycobacteria in humans were confused with those of tuberculosis. Therefore, methods used in routine laboratory diagnostics of tuberculosis should be mixed and include culture, Ziehl-Neelsen staining and at least one molecular technique. For culture isolation, growth media that will promote the growth of both *M. bovis* and *M. tuberculosis* should be used. The so called MOTT, referring to mycobacteria spp. other than the targeted known pathogenic *M. bovis* and *M. tuberculosis*, should no longer be disregarded and should be identified fully as they might be primary causative agents of some tuberculosis-like lesions or clinical conditions/cases in both animals and humans. Although the involvement of non-tuberculous mycobacteria in the disease-causing process has been established in humans and some animal species as well as their zoonotic aspects, this involvement still remains to be established in animals. Laboratories and researchers should also be encouraged to deposit newly identified mycobacterial spp. in the international gene data bank as there have been reports on unpublished data of new mycobacteria spp. in South Africa (Gcebe *et al*., 2013; Helden *et al*., 2009).

The emergence of MDR/XDR TB strains is of concern, and adding to the target of accurate identification of mycobacterial isolates, reference laboratories should implement a routine antimycobacterial sensitivity test for accurately identified isolates and perhaps also partially identified mycobacteria to allow a rapid implementation of effective treatment regimens. Annual reports may be compiled of antimycobacterial pattern sensitivity observed in the laboratory. As a long term goal, creating a national database on antimycobacterial sensitivity of mycobacterial isolates from humans and animals will be relevant as spill-over of *M. tuberculosis* from humans to animals has been reported and spill-back could also be a possibility (Michel *et al*., 2013; Ocepek *et al*., 2005). Medical and veterinary laboratories should collaborate on generating databases for antimycobacterial sensitivity of *M. bovis* and *M. tuberculosis* isolates. This task could be conducted in medical laboratories as tuberculosis in animals is not treated.

6.7.2 Antimycobacterial activities of leaf extracts, fractions and compounds isolated from plant species from the Anacardiaceae family

Besides the above, an ultimate goal is to continue to seek for alternative treatments exploring natural resources (plants) at our disposal for potential leads to anti-TB drug development. Natural products such as plant extracts, either as pure compounds or as standardized extracts, provide unlimited opportunities for new discoveries because of the unavailability of a chemical diversity database. A national database should be created and voucher samples of isolated anti-TB compounds deposited. This would allow at some stage easy traceability when networking with pharmaceutical companies in exploring the development of new drugs from targeted compounds.

Extraction is a crucial step because it is necessary to extract the desired chemical components from the plant material. The solvent used is equally important depending on the assay conducted. In the case of biological activity one must make sure that the solvent is not harmful to the microorganisms tested. Many solvents inhibit the growth of microorganisms. When evaluating the biological activity of extracts against microorganisms, acetone was proven not to inhibit the growth of tested microorganisms (Eloff, 1998a).

During this study, five plant species, namely *Harpephyllum caffrum*, *Protorhus longifolia*, *Searsia lancea*, *Searsia undulata* and *Sclerocarya birrea* had good antimycobacterial activity, and they will be further analysed. For the purpose of this study, one of the plants, *Searsia undulata,* contained about ten antimycobacterial compounds visible in bioautography. Of these, three compounds were isolated and two identified.

Bioassay-guided isolation of compounds is laborious and demanding. It needs a lot of patience and bioassays at each stage should be conducted scrupulously to identify the fractions in which active phytochemicals are present and to continue until isolation of the target compound(s). Several challenges were encountered during this study. Among others some compounds were unstable as demonstrated by changing TLC fingerprints. Compounds also appeared to have closely related structures making them difficult to separate. Adhering to certain environmental conditions from the extraction to isolation of compounds must be observed as some compounds are sensitive to heat; oxygen, temperature or light which might change their structure, therefore, avoiding exposure to these conditions will minimize structural changes.

6.7.3 *In vitro* cytotoxicity activity of *Searsia undulata* leaf extracts and isolated compounds against Vero and human hepatoma cell lines

In vitro cytotoxicity evaluation is important as compounds with fewer adverse effects are needed, considering that some of the current anti-TB drugs have undesirable side effects. When evaluating plants, the use of normal and abnormal cells (cancer cells) is relevant as cancer is a major cause of mortality and a cure is yet to be found for different types of cancer. Identifying potential antimicrobial and anticancer compounds can also be concurrently explored. This is the case during this study where an incidental finding revealed that *Searsia undulata* had high cytotoxicity to cancerous liver cells (C3A hepatoma cells) but low cytotoxicity to normal cells (Vero monkey kidney cells). This was supported by the compound isolated and identified as betulonic acid, which, together with its derivatives, has been reported to have anticancer, anti-inflammatory, antiviral and antimicrobial activities.

6.7.4 Future perspectives

As part of continued research relating to this project, there are many activities to be carried out in the future deriving from this study for publication purposes, and eventually drug development. The different activities recommended will be planned as short term, medium and long term goals.

6.7.4.1 Isolation and identification of compounds from Searsia undulata

Ten antimycobacterial compounds were observed on bioautography and two compounds were identified. One of the compounds, PK-B, requires further analysis to confirm beyond any doubt that it is indeed a novel compound. Crystallization, melting point and Fourier Transform Infrared spectroscopy are among the techniques needing to be done. The isolation and identification of the remainder of compounds will be carried out.

6.7.4.2 In vitro safety of all compounds isolated from S. undulata

The evaluation of the cytotoxicity of all isolated antimycobacterial compounds against at least three cell lines will be conducted.

6.7.4.3 Synergistic evaluation of compounds isolated from S. undulata

The synergy of antimycobacterial activity among the compounds and mixed with standard anti-TB drugs will be investigated.

6.7.4.4 Determine the stability of each compound and fractions of S. undulata

Observations made during this study indicate that some compounds might be unstable; therefore, it is important to evaluate the stability of each compound as a labile compound may not be useful. The stability in solution and as powder under different conditions of temperature, moisture, light, and presence or absence of oxygen needs to be determined.

6.7.4.5 Determine antioxidant, anti-inflammatory and inhibition of nitric oxide production by fractions and compounds of S. undulata

Testing for antioxidant activity and anti-inflammatory activities should be conducted. Inflammation, which evolves into chronic inflammation, as well as the production of oxidative radicals, takes place during the pathogenesis of acute or chronic tuberculosis.

6.7.4.6 Determine the in vivo safety of pure compounds

Identifying an animal model for *in vivo* evaluation of efficacy is an area that requires attention in the future. Clinical trials are also necessary to demonstrate the effectiveness and safety of the bioactive compounds. In the course of drug development, determining acute and chronic toxicity is required.

6.7.4.7 Develop an herbal product from S. undulata

Considering that the development of drugs is expensive, one could first investigate in the local communities the current ethnobotanical use of this plant species. The selection of species in this study was extended to the Anacardiaceae family based on few members of the family that had good antibacterial activities on Gram-positive and Gram-negative bacteria as well as *M. smegmatis* in a previous study (Pauw, 2004). It was subsequently discovered in the literature that leaves are chewed by the Khoi San to treat chest colds and this needs further investigation. A survey in this population to find out about any other information on the ethnobotanical use and observations about this plant species could be initiated.

6.7.4.8 Understanding the mechanism of actions of compounds

In the case of *S. undulata*, leaves are chewed to treat chest colds. Each group of currently-used antibiotics has a different mechanism of action - bactericidal or bacteriostatic on microorganisms. The emergence of MDR-TB drugs is of concern and therefore focusing on mechanisms of action to prevent rapid development of resistance is crucial. There is evidence that the development of resistance may be minimized when using plant extracts, as a combination of different compounds may exert a range of different activities. In the case of tuberculosis, mycobacteria might be present in the latent “dormant” phase, and can be reactivated when immunity is compromised.

Therefore the best drug or combination of drugs should be bactericidal towards the mycobacteria, particularly those inside macrophages. Drugs with additional positive effects on the immune system are also desirable.

6.7.4.9 Develop network with pharmaceutical companies

Establishing network with companies interested in tuberculosis drugs will be part of the long term goal of this research.

6.7.4.10 Testing of four other plant species with good antimycobacterial activity

The other plant species (*Harpephyllum caffrum*, *Protorhus longifolia*, *Searsia lancea* and *Sclerocarya birrea*) that have good antimycobacterial activity will be further processed as mentioned under all the above sections.

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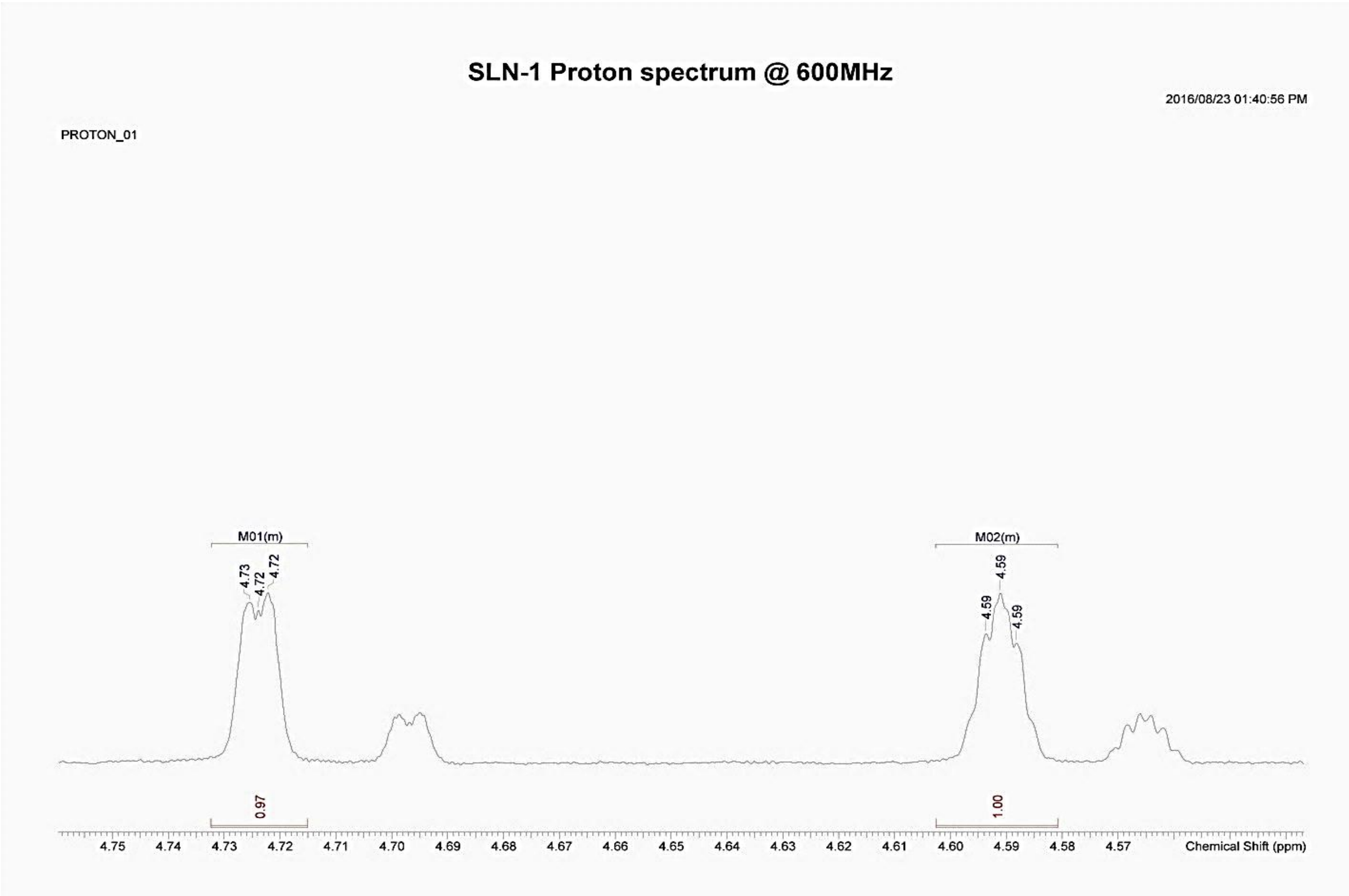
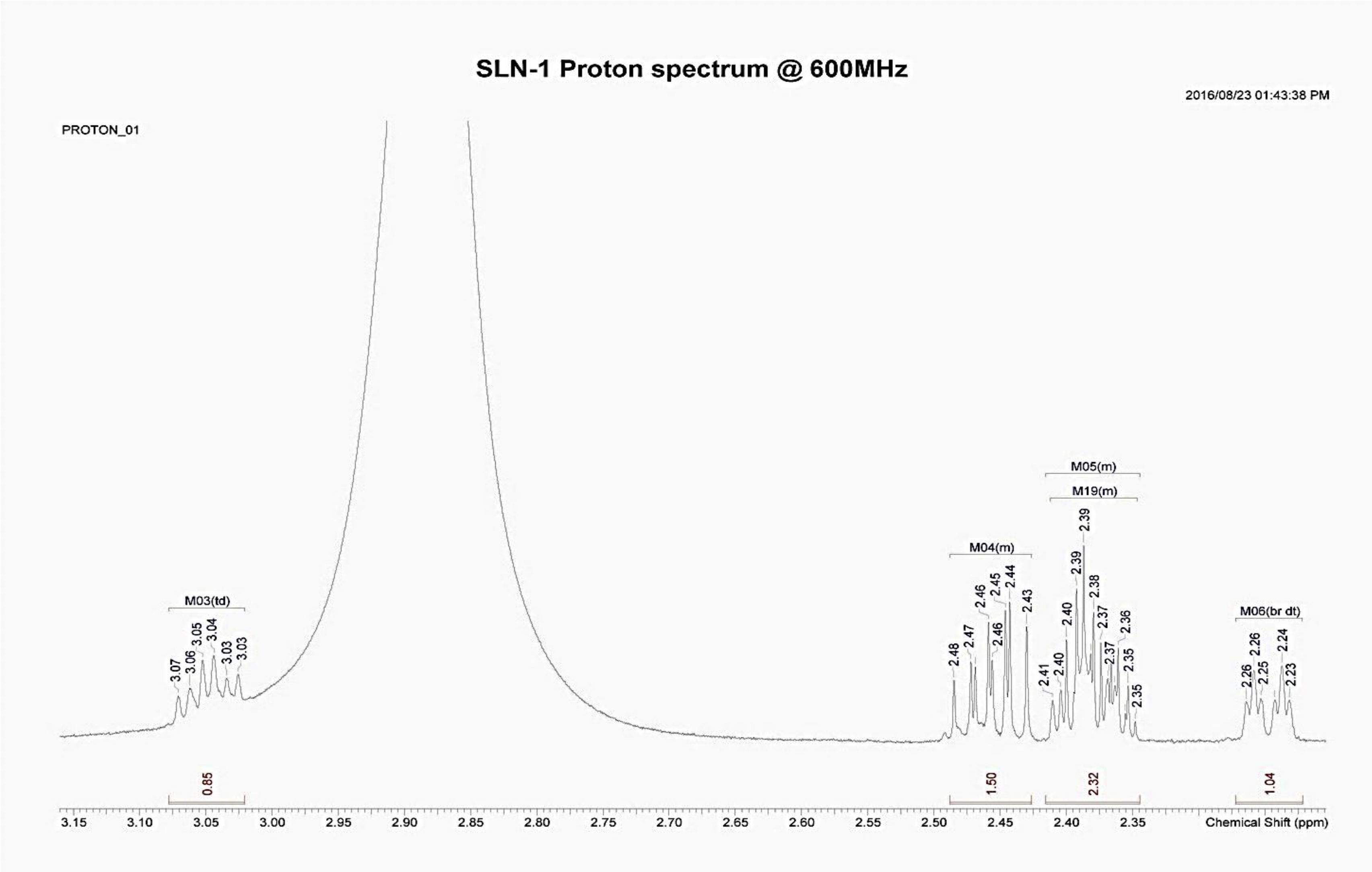
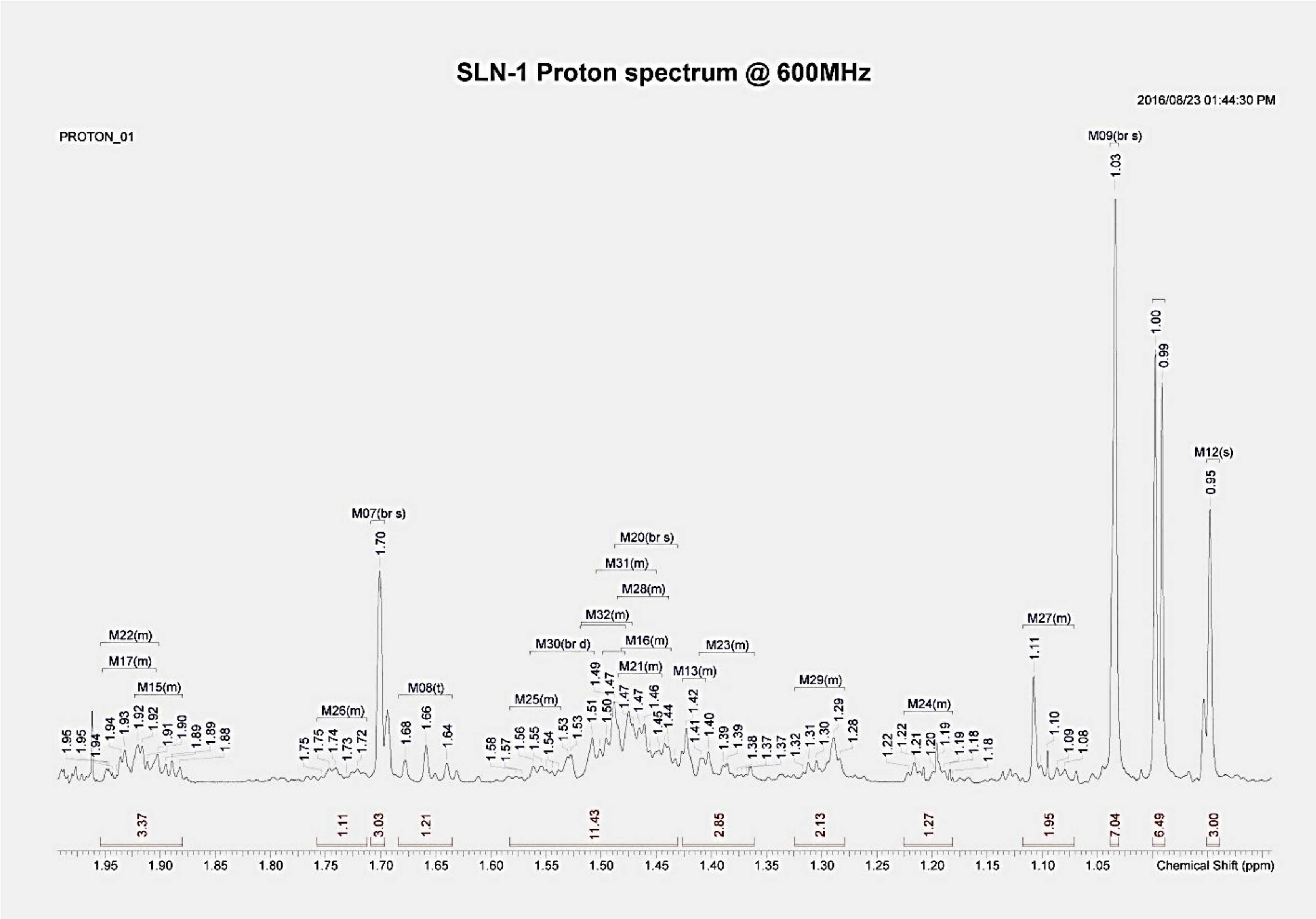
APPENDICES

Appendix A: Structure elucidation of compound SLN-1




SLN-1 Proton spectrum @ 600MHz
2016/08/23 01:43:38 PM
PROTON_01
M03(td)
M04(m)
M05(m)
M19(m)
M06(br dt)
0.85
1.50
2.32
1.04
Chemical Shift (ppm)



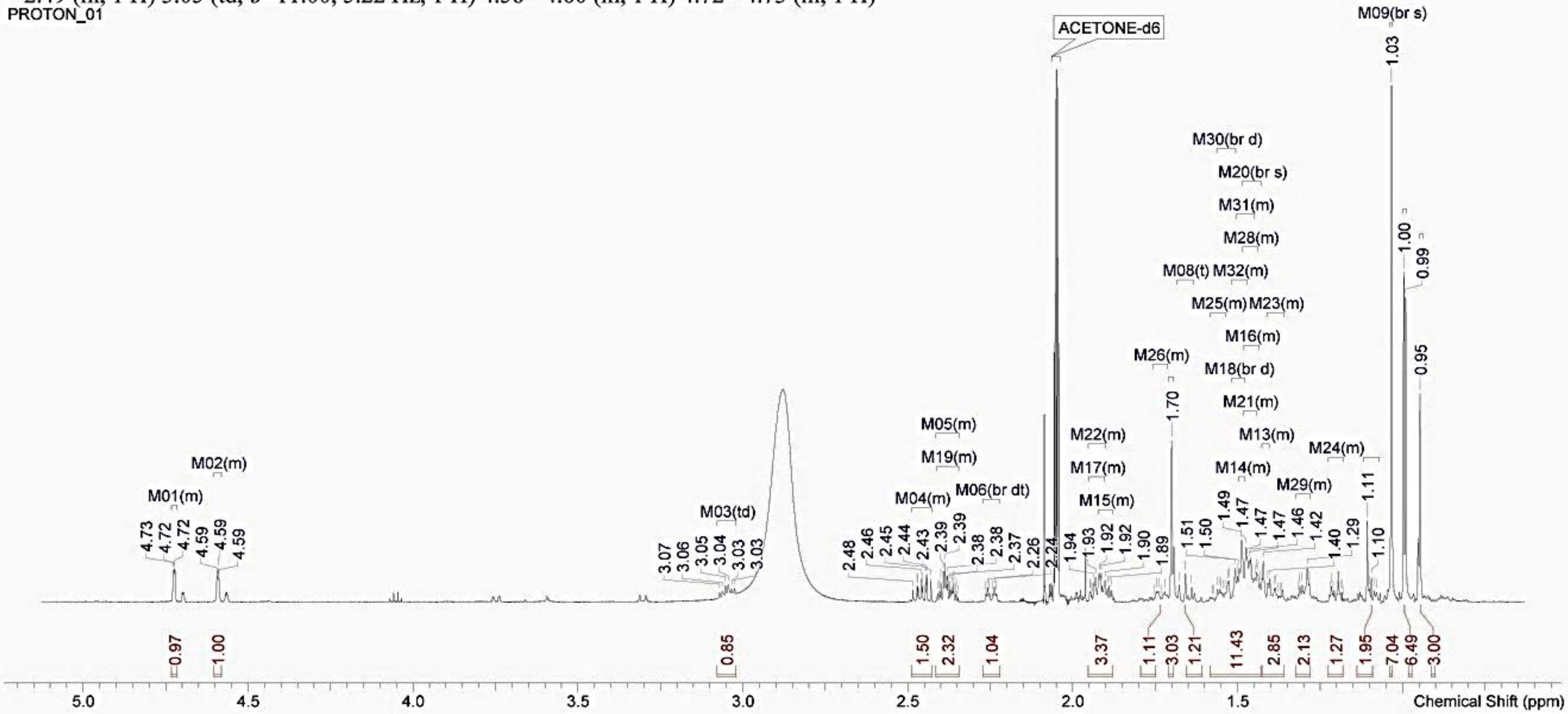
SLN-1 Proton spectrum @ 600MHz
2016/08/23 01:44:30 PM
PROTON_01
M09(br s)
M12(s)
M07(br s)
M20(br s)
M31(m)
M28(m)
M32(m)
M22(m)
M17(m)
M15(m)
M30(br d)
M16(m)
M23(m)
M21(m)
M13(m)
M08(t)
M25(m)
M26(m)
M29(m)
M24(m)
M27(m)
Chemical Shift (ppm)

SLN-1 Proton spectrum @ 600MHz

2016/08/23 01:38:43 PM

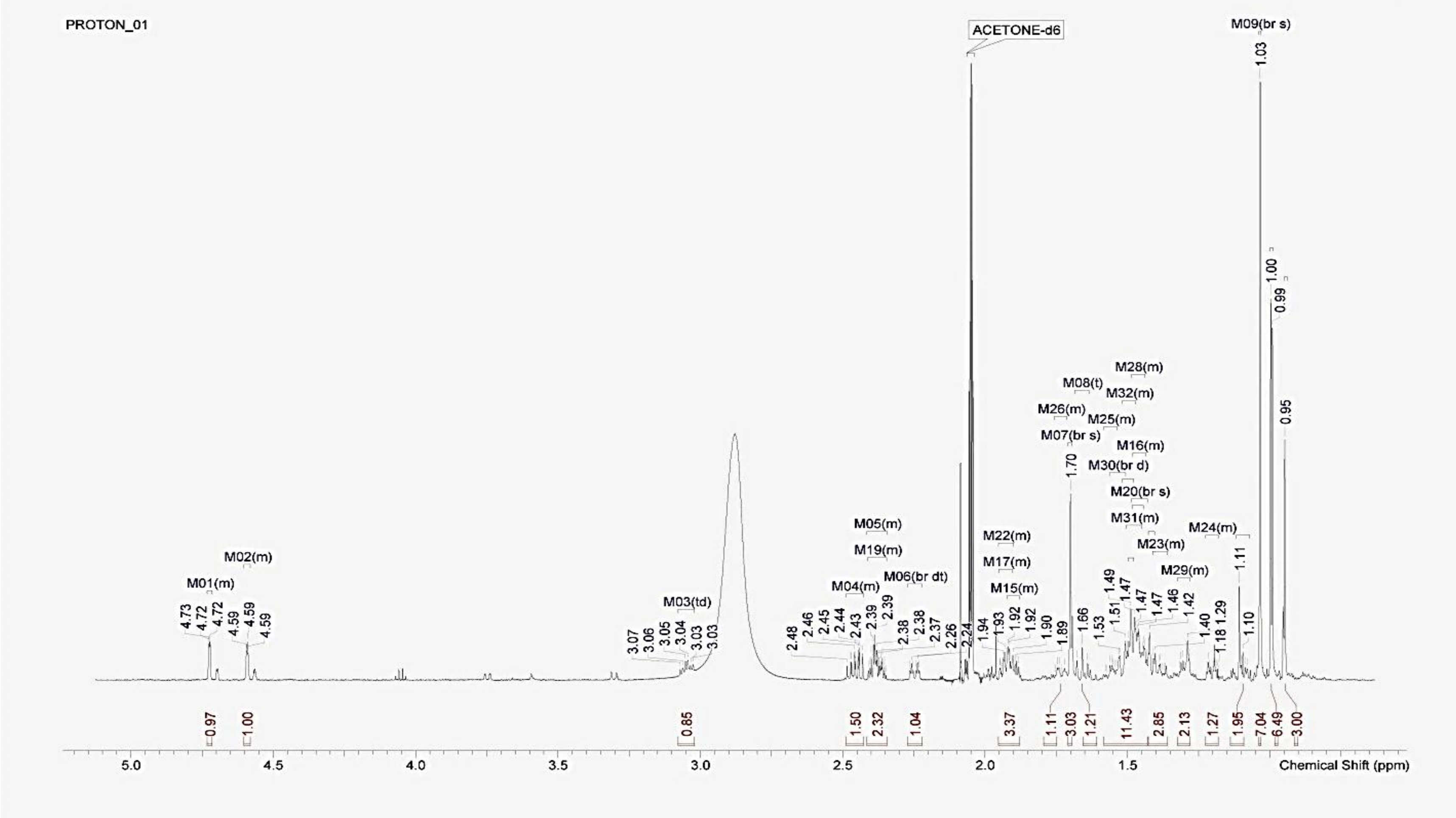
Acquisition Time (sec)	1.7042	*Date*	Mar 22 2016			*Date Stamp*	Mar 22 2016		
File Name	G:\SLN\SNL-1_20160322_01\PROTON_01.fid\fid			*Frequency (MHz)*	599.7405	*Nucleus*	1H	*Number of Transients*	1
Original Points Count	4591	*Points Count*	32768	*Pulse Sequence*	s2pul	*Receiver Gain*	34.00	*SW(cyclical) (Hz)*	2693.97
Solvent	acetone	*Spectrum Offset (Hz)*	1726.1036	*Spectrum Type*	standard	*Sweep Width (Hz)*	2693.88	*Temperature (degree C)*	30.000

^{1}H NMR (600 MHz, Solvent) δ ppm 0.95 (s, 3 H) 0.99 (d, J=3.70 Hz, 6 H) 1.03 (br s, 3 H) 1.03 - 1.04 (m, 3 H) 1.07 - 1.12 (m, 2 H) 1.18 - 1.23 (m, 1 H) 1.28 - 1.32 (m, 2 H) 1.36 - 1.41 (m, 2 H) 1.41 - 1.43 (m, 1 H) 1.43 (br s, 2 H) 1.44 - 1.48 (m, 1 H) 1.44 - 1.49 (m, 1 H) 1.45 - 1.48 (m, 1 H) 1.45 - 1.51 (m, 2 H) 1.47 - 1.52 (m, 1 H) 1.50 (br d, J=4.11 Hz, 1 H) 1.48 - 1.50 (m, 1 H) 1.53 (br d, J=1.81 Hz, 2 H) 1.54 - 1.58 (m, 1 H) 1.66 (t, J=11.30 Hz, 1 H) 1.70 (br s, 3 H) 1.71 - 1.76 (m, 1 H) 1.88 - 1.92 (m, 1 H) 1.90 - 1.95 (m, 1 H) 1.90 - 1.95 (m, 1 H) 2.25 (br dt, J=12.80, 3.30 Hz, 1 H) 2.34 - 2.42 (m, 1 H) 2.35 - 2.41 (m, 1 H) 2.43 - 2.49 (m, 1 H) 3.05 (td, J=11.00, 5.22 Hz, 1 H) 4.58 - 4.60 (m, 1 H) 4.72 - 4.73 (m, 1 H)

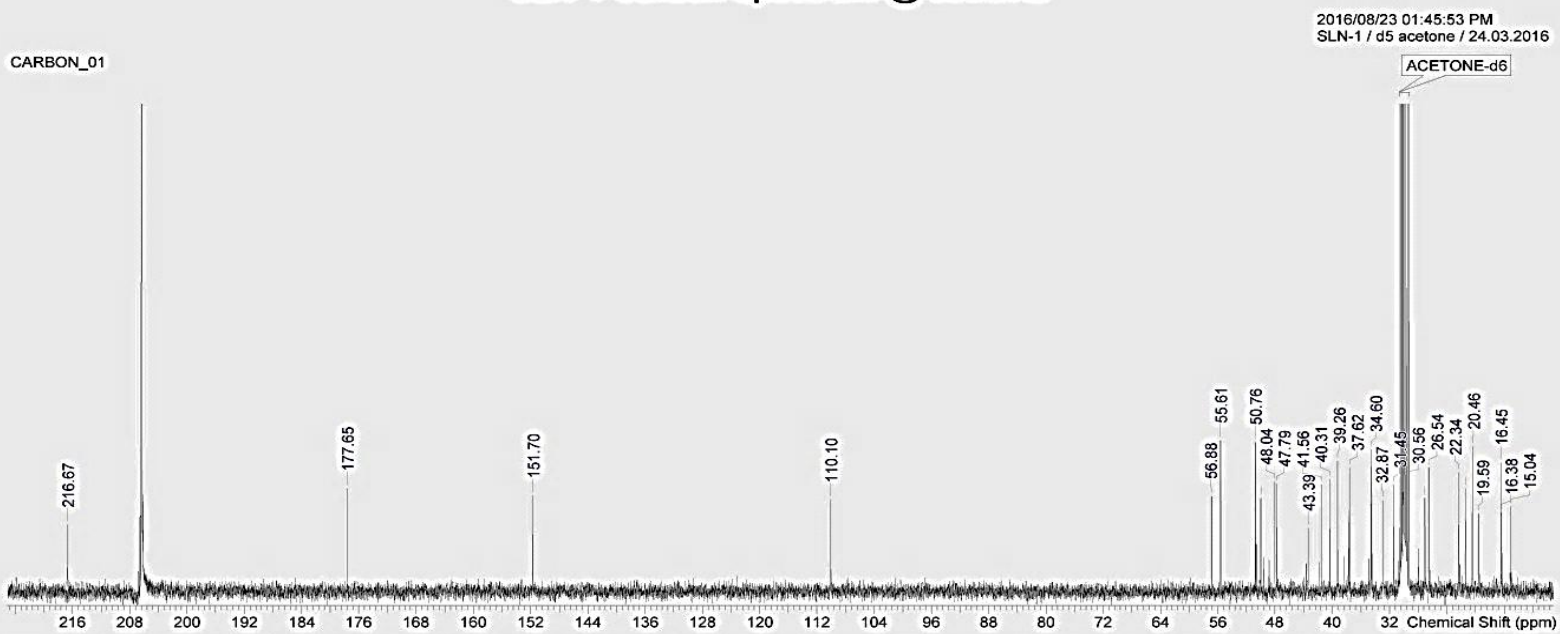


SLN-1 Proton spectrum @ 600MHz

2016/08/23 01:42:26 PM



SLN-1 Carbon spectrum @ 600MHz

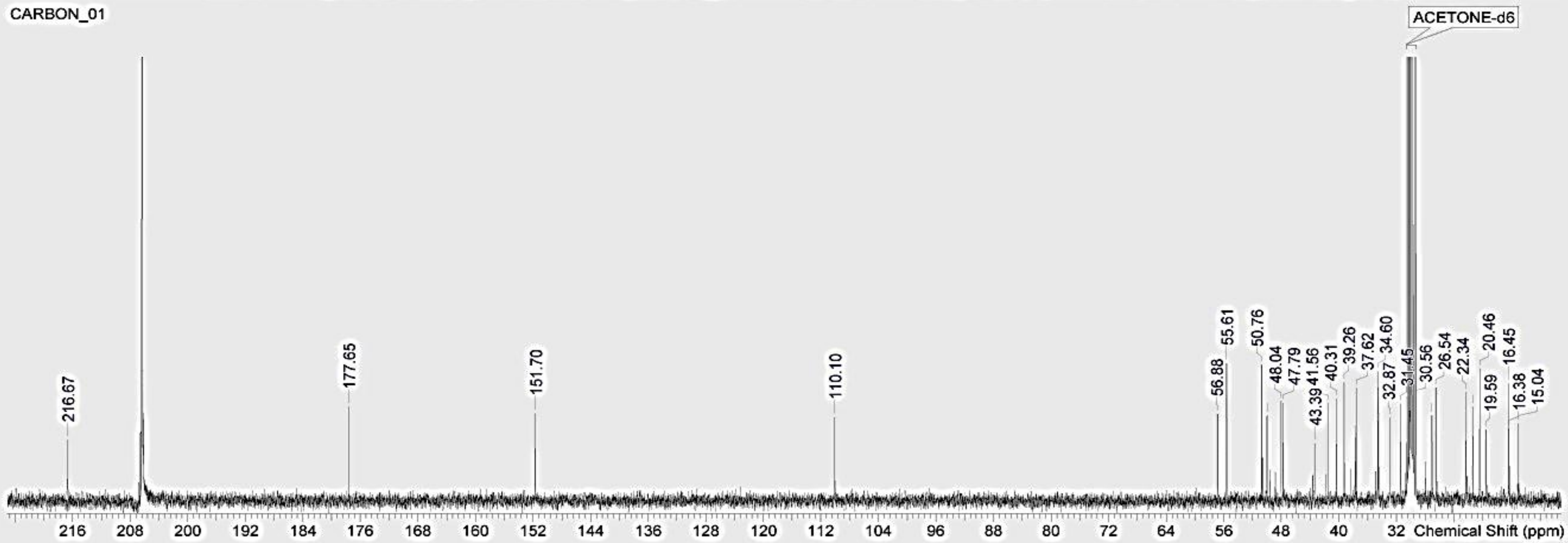


No.	(ppm)	Height	No.	(ppm)	Height	No.	(ppm)	Height	No.	(ppm)	Height	No.	(ppm)	Height	No.	(ppm)	Height
1	15.04	0.0166	6	21.40	0.0202	11	31.45	0.0209	16	37.76	0.0137	21	47.79	0.0211	26	56.88	0.0187
2	16.38	0.0156	7	22.34	0.0234	12	32.87	0.0179	17	39.26	0.0256	22	48.04	0.0215	27	110.10	0.0180
3	16.45	0.0253	8	26.54	0.0244	13	34.54	0.0245	18	40.31	0.0220	23	50.01	0.0184	28	151.70	0.0189
4	19.59	0.0153	9	27.09	0.0184	14	34.60	0.0279	19	41.56	0.0210	24	50.76	0.0293	29	177.65	0.0202
5	20.46	0.0285	10	30.56	0.0217	15	37.62	0.0243	20	43.39	0.0124	25	55.61	0.0297	30	216.67	0.0132

SLN-1 Carbon spectrum @ 600MHz

2016/08/23 01:46:34 PM
SLN-1 / d5 acetone / 24.03.2016

Acquisition Time (sec)	1.3578	*Comment*	SLN-1 / d5 acetone / 24.03.2016			*Date*	Mar 24 2016	*Date Stamp*	Mar 24 2016
File Name	G:\SLN\SLN-1_20160324_01\CARBON_01.fid\fid			*Frequency (MHz)*	100.5760	*Nucleus*	13C	*Number of Transients*	5000
Original Points Count	32768	*Points Count*	32768	*Pulse Sequence*	s2pul	*Receiver Gain*	58.00	*SW(cyclical) (Hz)*	24132.73
Solvent	acetone	*Spectrum Offset (Hz)*	11668.5703	*Spectrum Type*	standard	*Sweep Width (Hz)*	24132.73	*Temperature (degree C)*	30.000



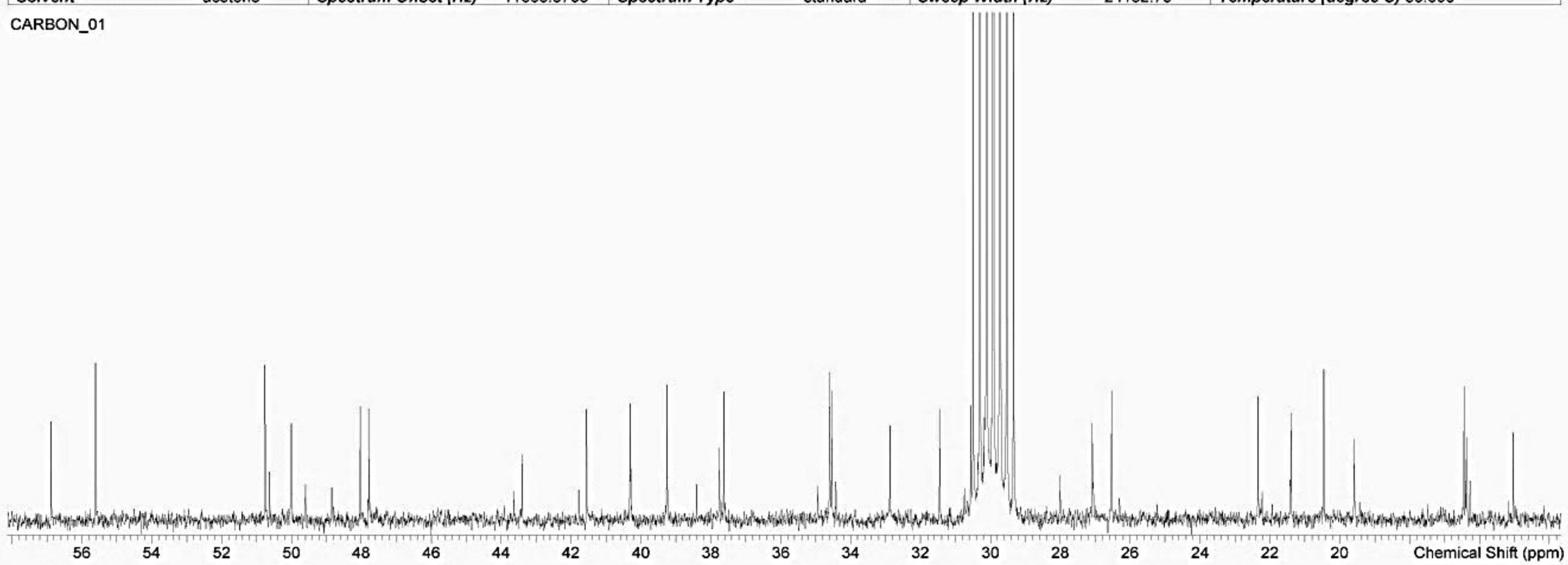
No.	(ppm)	Height	No.	(ppm)	Height	No.	(ppm)	Height	No.	(ppm)	Height	No.	(ppm)	Height	No.	(ppm)	Height
1	15.04	0.0166	6	21.40	0.0202	11	31.45	0.0209	16	37.76	0.0137	21	47.79	0.0211	26	56.88	0.0187
2	16.38	0.0156	7	22.34	0.0234	12	32.87	0.0179	17	39.26	0.0256	22	48.04	0.0215	27	110.10	0.0180
3	16.45	0.0253	8	26.54	0.0244	13	34.54	0.0245	18	40.31	0.0220	23	50.01	0.0184	28	151.70	0.0189
4	19.59	0.0153	9	27.09	0.0184	14	34.60	0.0279	19	41.56	0.0210	24	50.76	0.0293	29	177.65	0.0202
5	20.46	0.0285	10	30.56	0.0217	15	37.62	0.0243	20	43.39	0.0124	25	55.61	0.0297	30	216.67	0.0132

SLN-1 Carbon spectrum @ 600MHz

2016/08/23 01:47:08 PM
SLN-1 / d5 acetone / 24.03.2016

Acquisition Time (sec)	1.3578	***Comment***	SLN-1 / d5 acetone / 24.03.2016	***Date***	Mar 24 2016	***Date Stamp***	Mar 24 2016
File Name	G:\SLN\SLN-1_20160324_01\CARBON_01.fid\fid	***Frequency (MHz)***	100.5760	***Nucleus***	13C	***Number of Transients***	5000
Original Points Count	32768	***Points Count***	32768	***Receiver Gain***	58.00	***SW(cyclical) (Hz)***	24132.73
Solvent	acetone	***Spectrum Offset (Hz)***	11668.5703	***Sweep Width (Hz)***	24132.73	***Temperature (degree C)***	30.000

Pulse Sequence	s2pul
Spectrum Type	standard



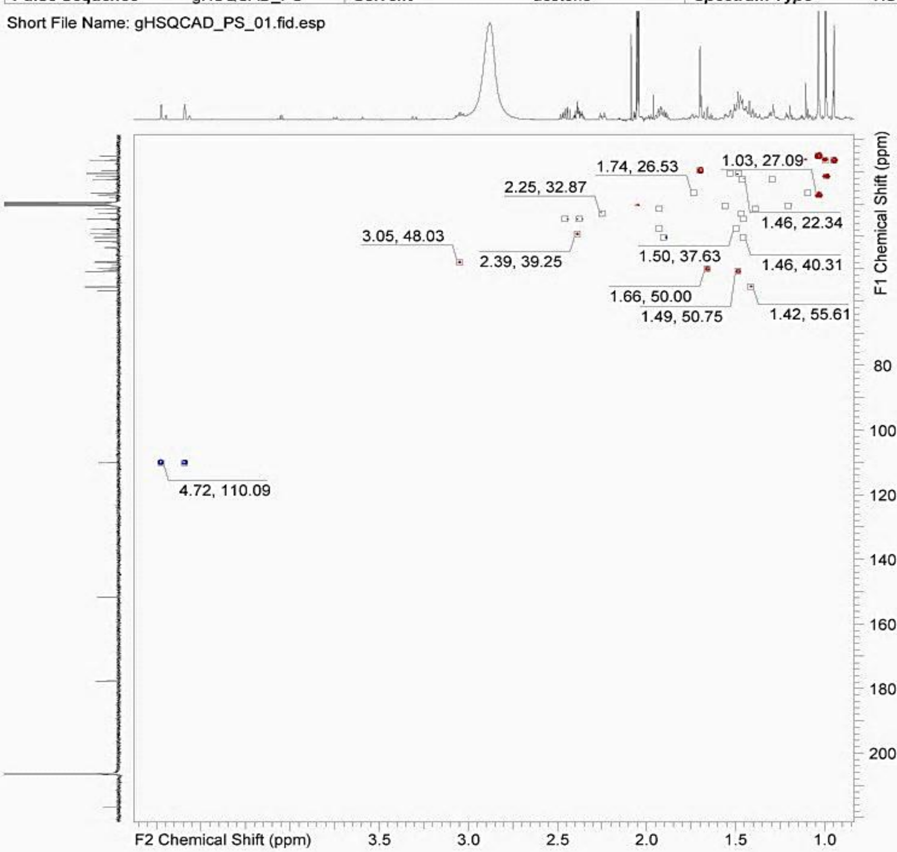
No.	(ppm)	Height	No.	(ppm)	Height	No.	(ppm)	Height	No.	(ppm)	Height	No.	(ppm)	Height	No.	(ppm)	Height
1	15.04	0.0166	6	21.40	0.0202	11	31.45	0.0209	16	37.76	0.0137	21	47.79	0.0211	26	56.88	0.0187
2	16.38	0.0156	7	22.34	0.0234	12	32.87	0.0179	17	39.26	0.0256	22	48.04	0.0215	27	110.10	0.0180
3	16.45	0.0253	8	26.54	0.0244	13	34.54	0.0245	18	40.31	0.0220	23	50.01	0.0184	28	151.70	0.0189
4	19.59	0.0153	9	27.09	0.0184	14	34.60	0.0279	19	41.56	0.0210	24	50.76	0.0293	29	177.65	0.0202
5	20.46	0.0285	10	30.56	0.0217	15	37.62	0.0243	20	43.39	0.0124	25	55.61	0.0297	30	216.67	0.0132

SLN-1 gHSQC-PS @ 600MHz

23 Aug 2016

Acquisition Time (sec)	(0.2487, 0.0179)	***Date***	29 Mar 2016 11:04:18			***Date Stamp***	Mar 22 2016
File Name	G:\SLN\SNL-1_20160322_01\gHSQCAD_PS_01.fid\fid			***Frequency (MHz)***	(599.7388, 150.8044)		
Nucleus	(1H, 13C)	***Number of Transients***	16	***Original Points Count***	(670, 512)	***Points Count***	(1024, 2048)
Pulse Sequence	gHSQCAD_PS	***Solvent***	acetone	***Spectrum Type***	HSQC-DEPT	***Sweep Width (Hz)***	(2691.33, 28639.30)

Short File Name: gHSQCAD_PS_01.fid.esp

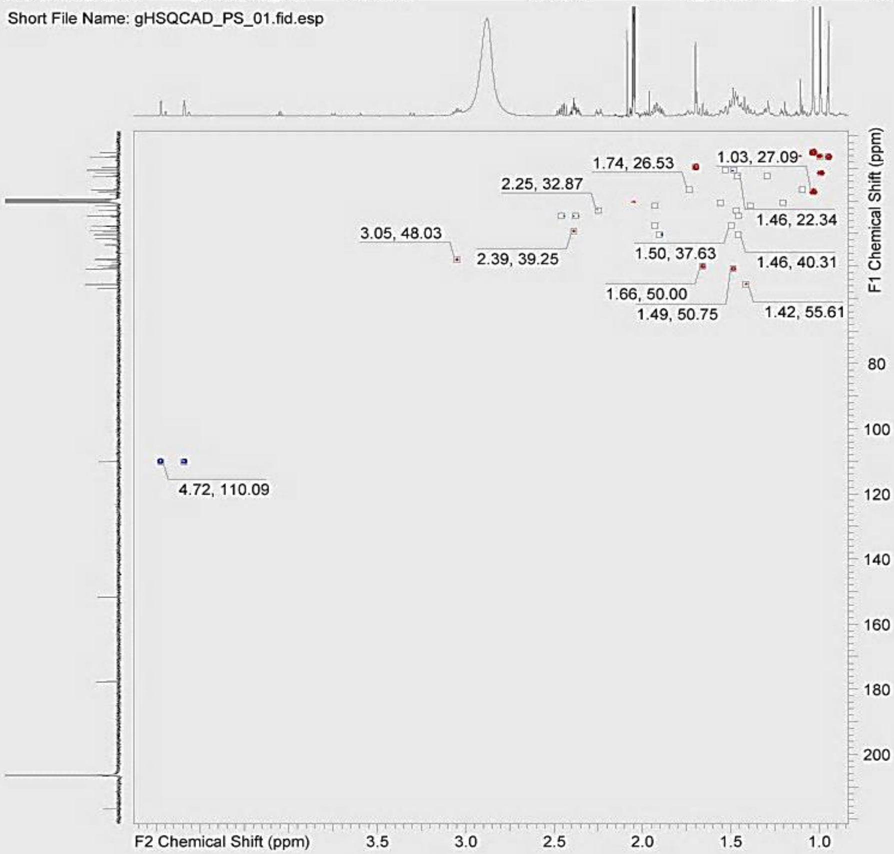


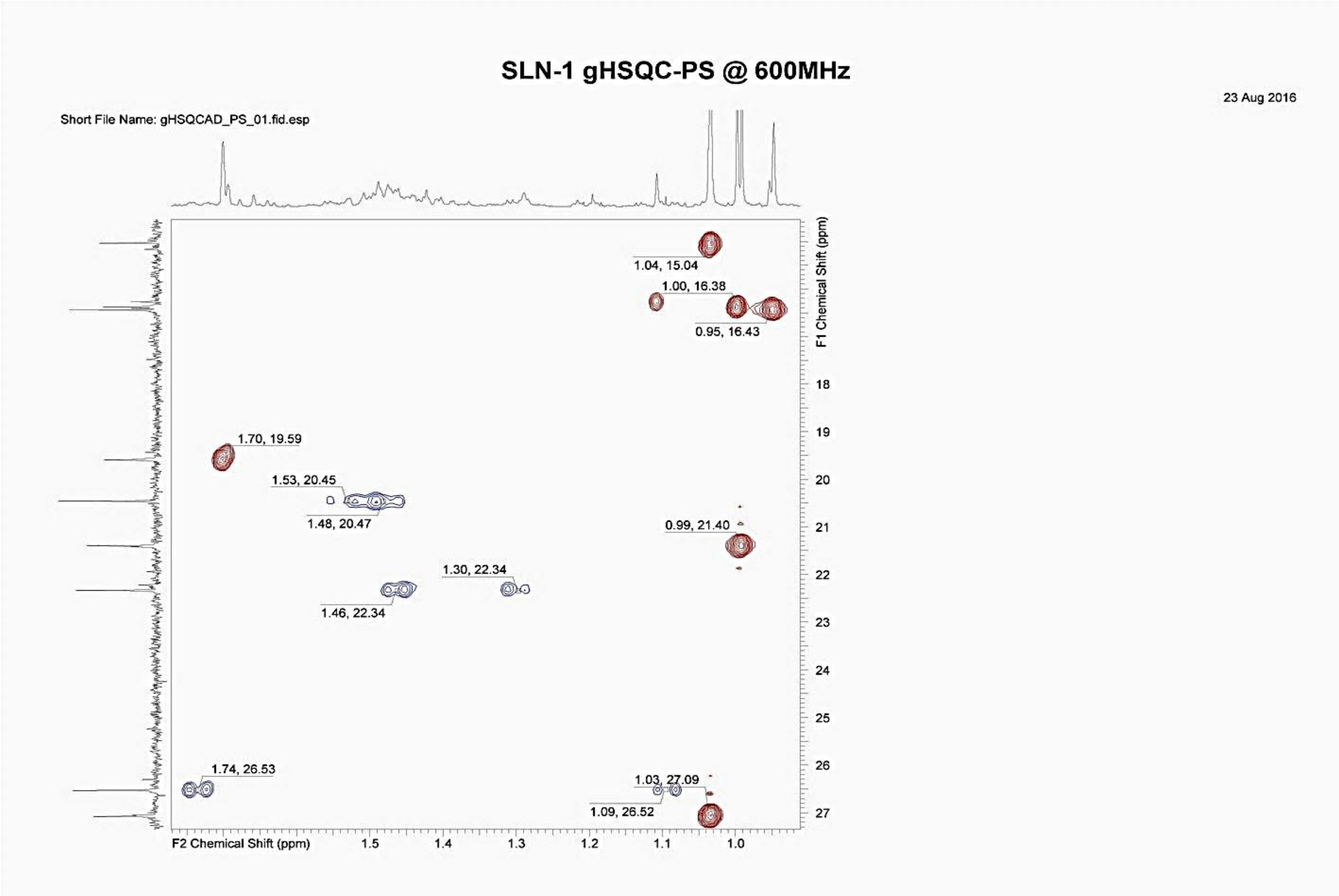
SLN-1 gHSQC-PS @ 600MHz

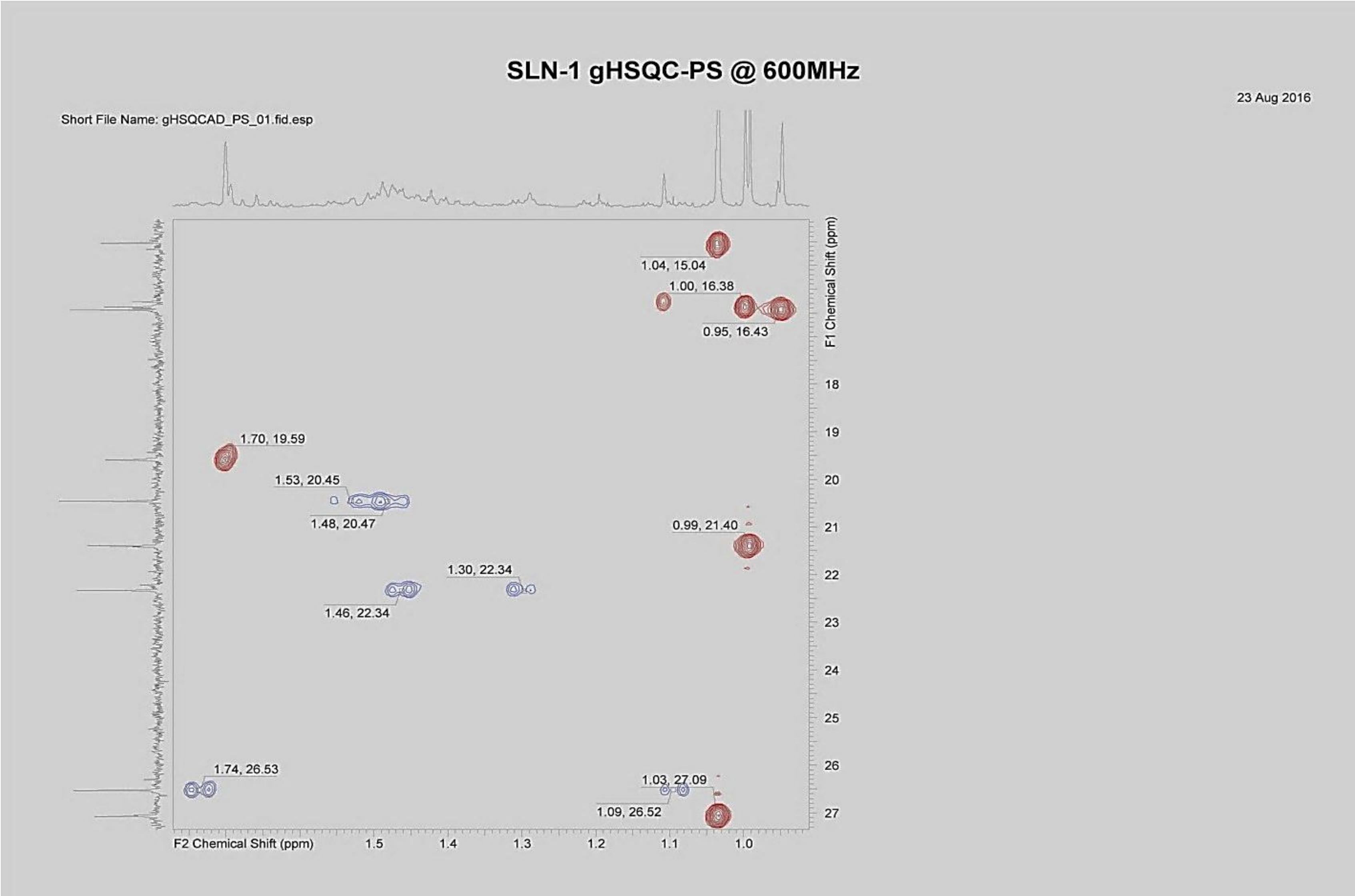
23 Aug 2016

Acquisition Time (sec)	(0.2487, 0.0179)	**Date**	29 Mar 2016 11:04:18			**Date Stamp**	Mar 22 2016
File Name	G:\SLN\SNL-1_20160322_01\gHSQCAD_PS_01.fid\fid			**Frequency (MHz)**	(599.7388, 150.8044)		
Nucleus	(1H, 13C)	**Number of Transients**	16	**Original Points Count**	(670, 512)	**Points Count**	(1024, 2048)
Pulse Sequence	gHSQCAD_PS	**Solvent**	acetone	**Spectrum Type**	HSQC-DEPT	**Sweep Width (Hz)**	(2691.33, 28639.30)

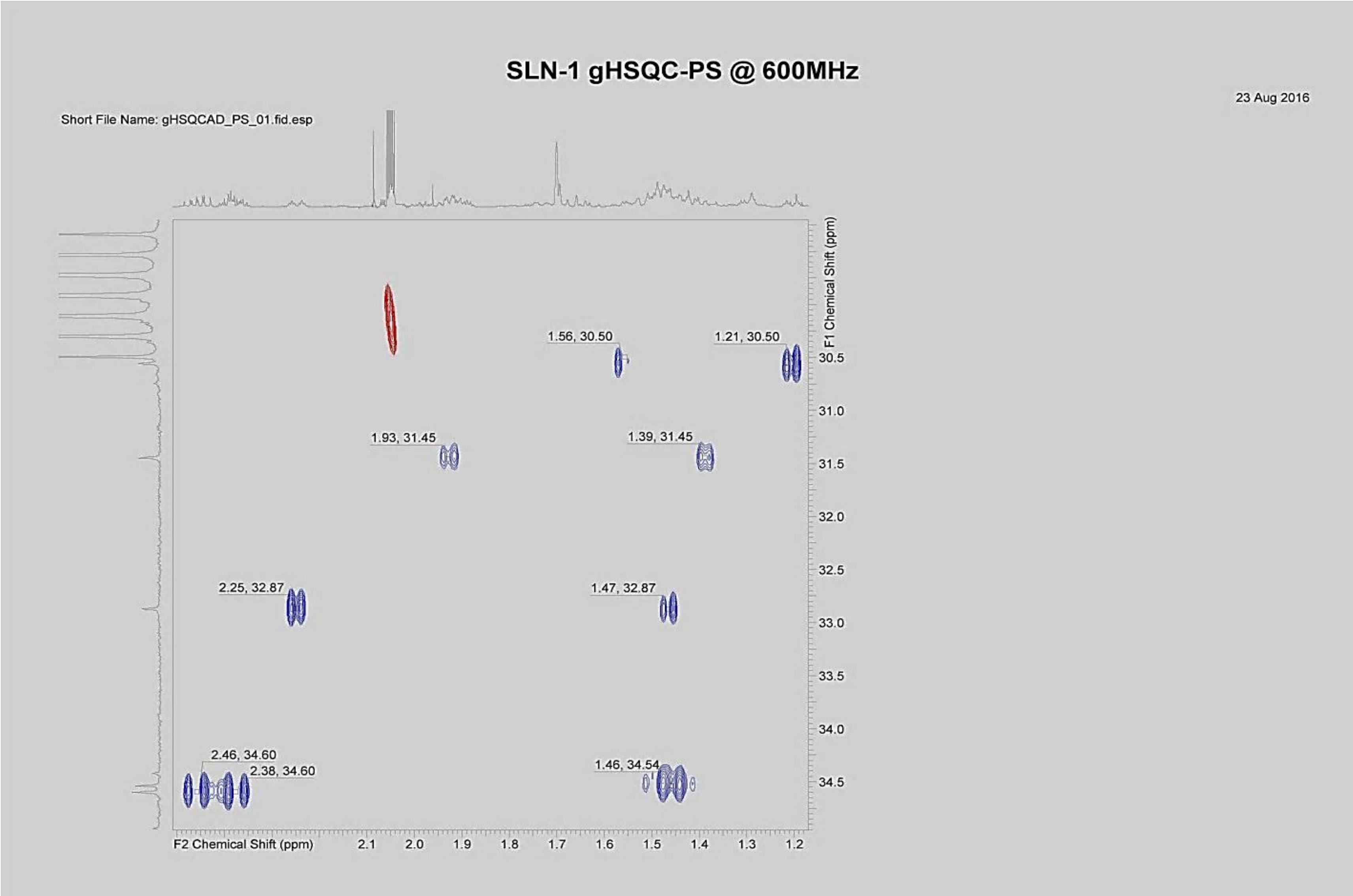
Short File Name: gHSQCAD_PS_01.fid.esp

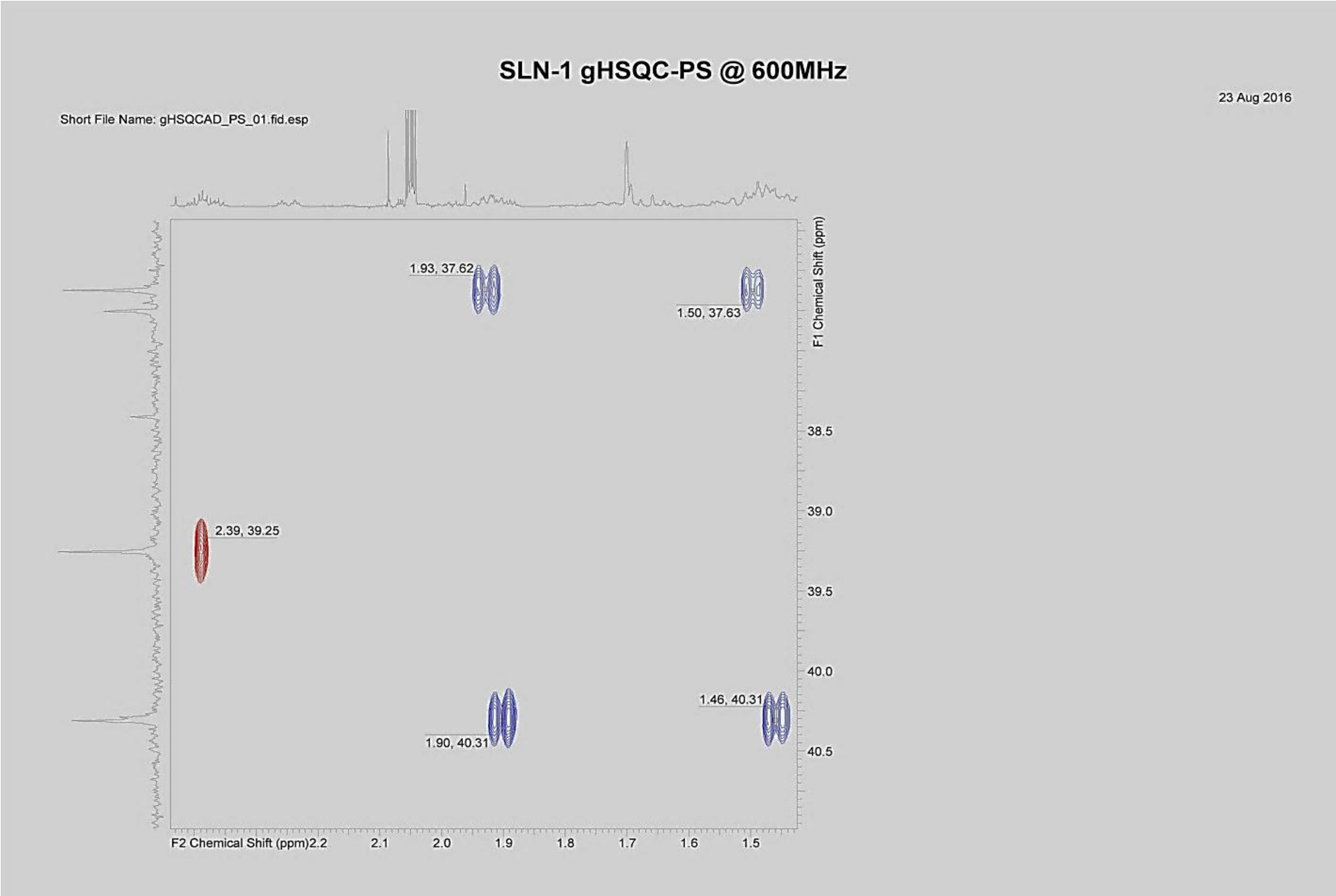



SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHSQCAD_PS_01.fid.esp
1.04, 15.04
1.00, 16.38
0.95, 16.43
1.70, 19.59
1.53, 20.45
1.48, 20.47
0.99, 21.40
1.30, 22.34
1.46, 22.34
1.74, 26.53
1.03, 27.09
1.09, 26.52
F1 Chemical Shift (ppm)
F2 Chemical Shift (ppm)
18
19
20
21
22
23
24
25
26
27
1.5
1.4
1.3
1.2
1.1
1.0

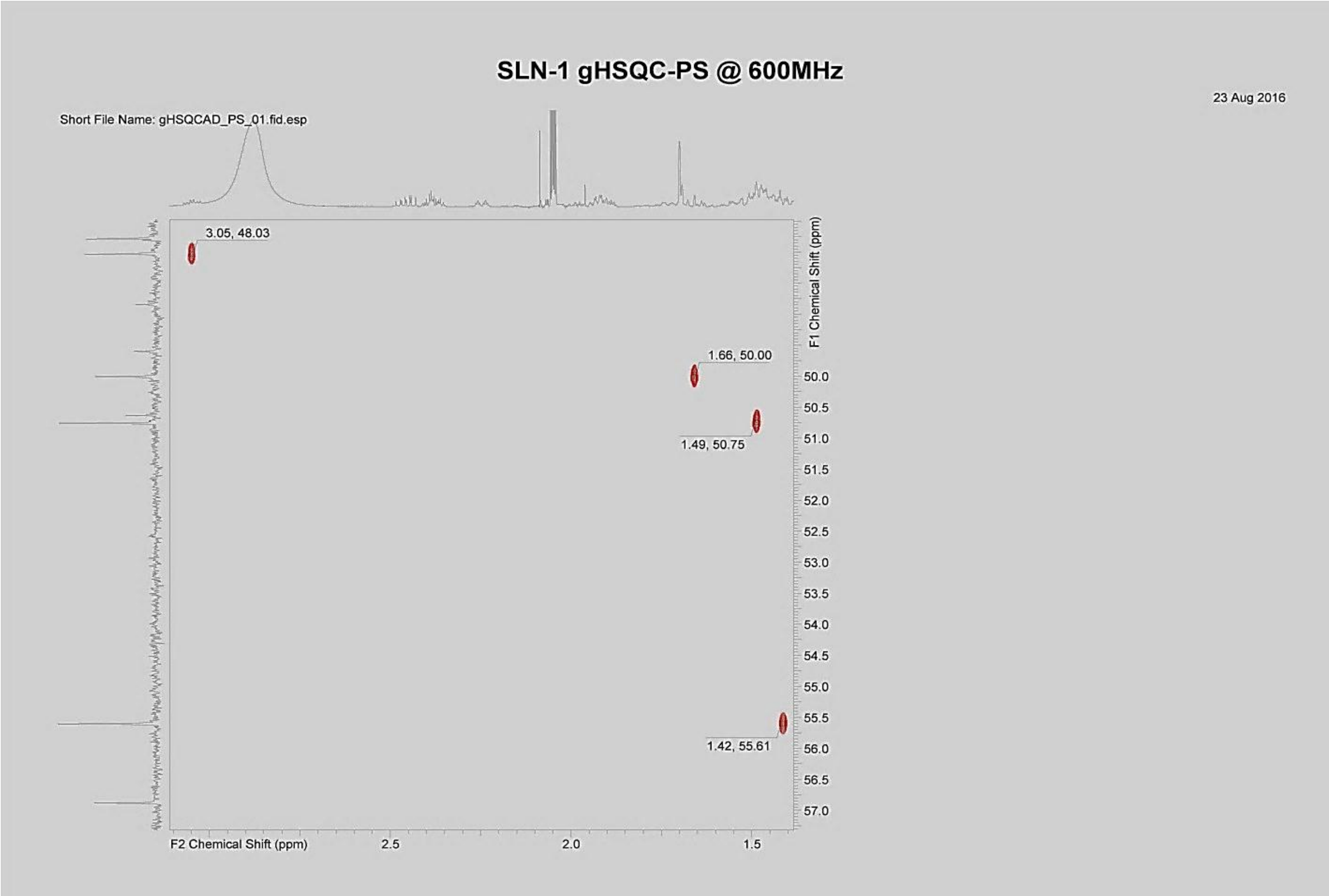


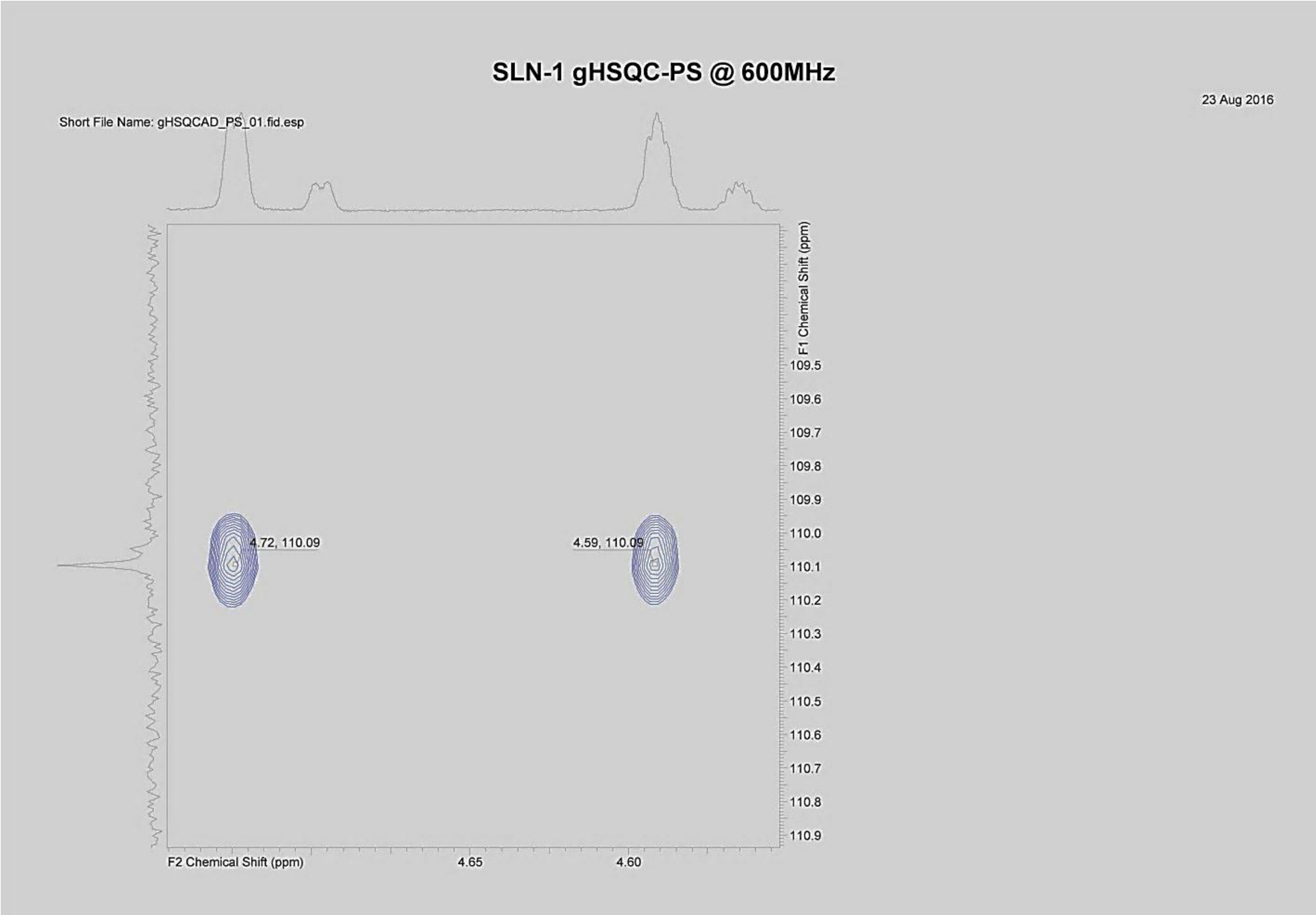
SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHSQCAD_PS_01.fid.esp
1.04, 15.04
1.00, 16.38
0.95, 16.43
1.70, 19.59
1.53, 20.45
1.48, 20.47
0.99, 21.40
1.30, 22.34
1.46, 22.34
1.74, 26.53
1.03, 27.09
1.09, 26.52
F1 Chemical Shift (ppm)
F2 Chemical Shift (ppm)

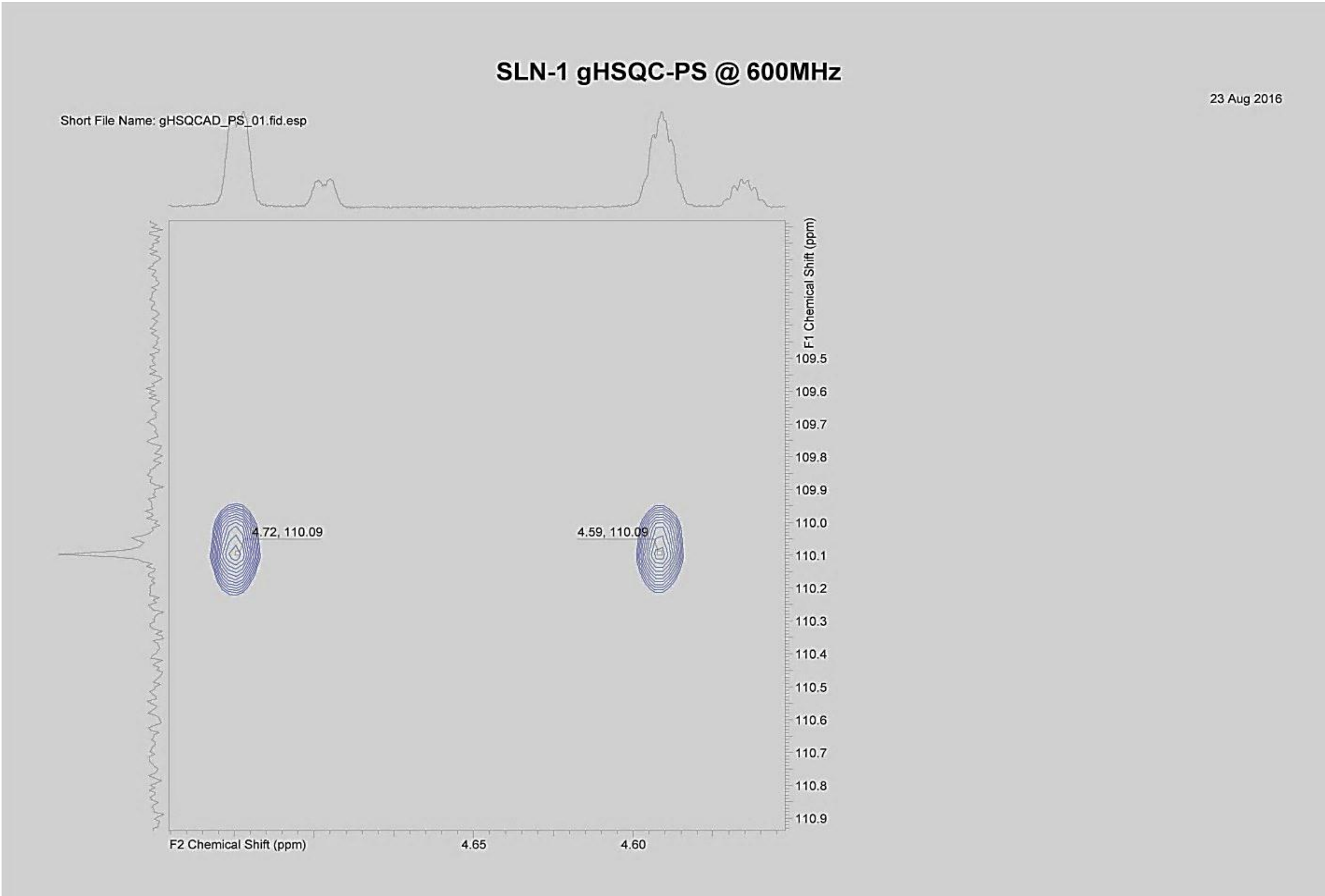

SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHSQCAD_PS_01.fid.esp
1.56, 30.50
1.21, 30.50
1.93, 31.45
1.39, 31.45
2.25, 32.87
1.47, 32.87
2.46, 34.60
2.38, 34.60
1.46, 34.54
F1 Chemical Shift (ppm)
30.5
31.0
31.5
32.0
32.5
33.0
33.5
34.0
34.5
F2 Chemical Shift (ppm)
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2

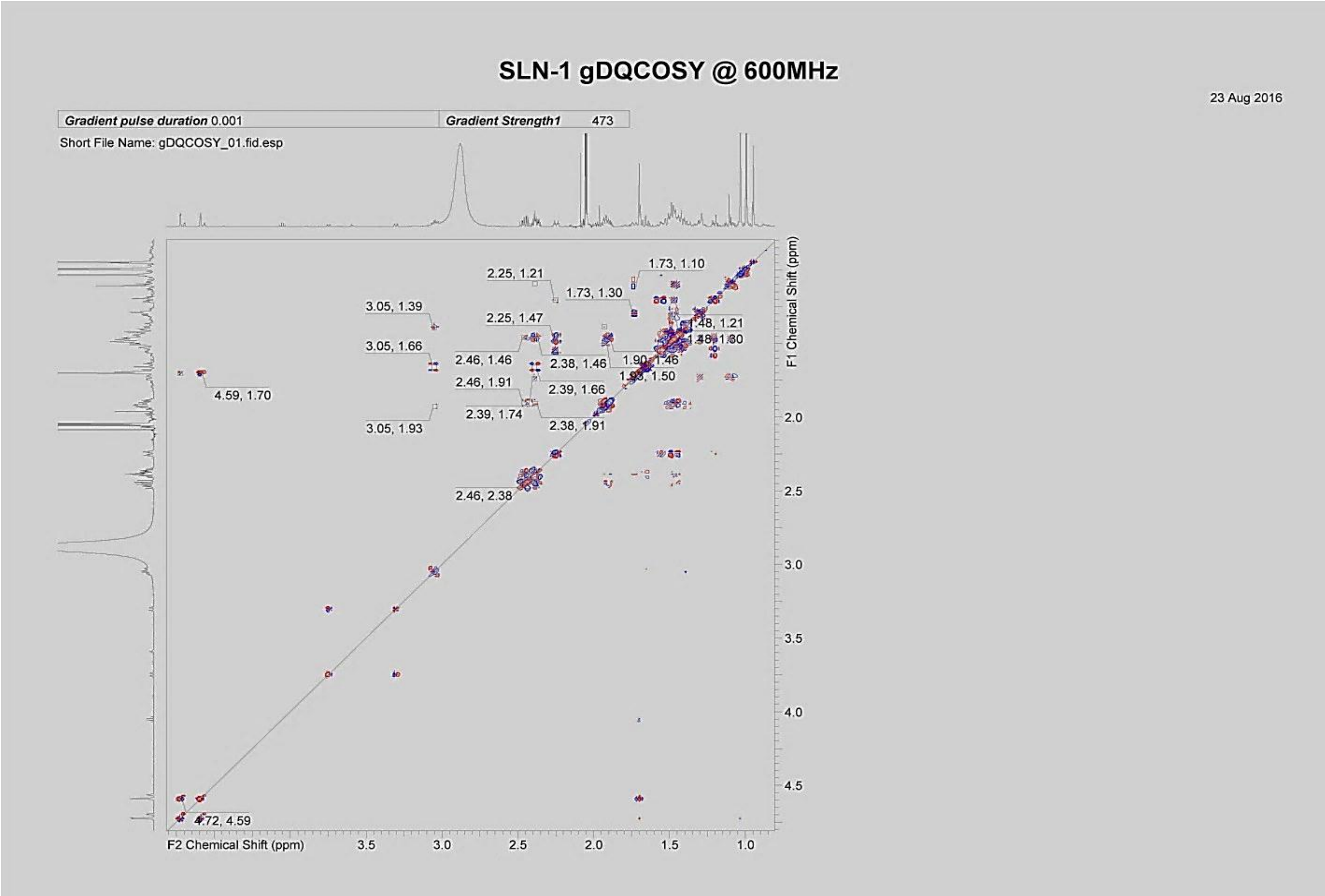


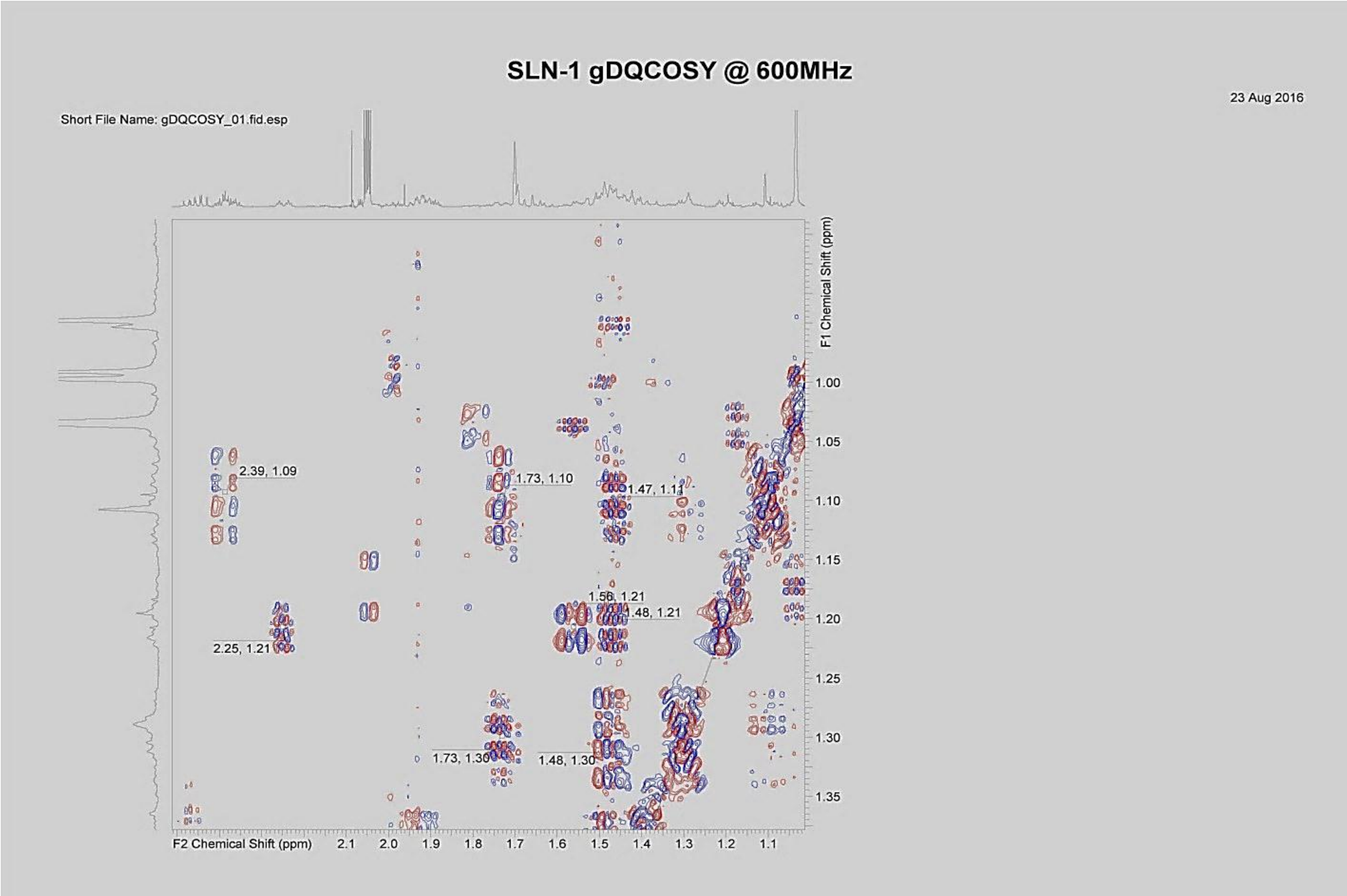
SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHSQCAD_PS_01.fid.esp
1.93, 37.62
1.50, 37.63
2.39, 39.25
1.46, 40.31
1.90, 40.31
F1 Chemical Shift (ppm)
38.5
39.0
39.5
40.0
40.5
F2 Chemical Shift (ppm)2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5

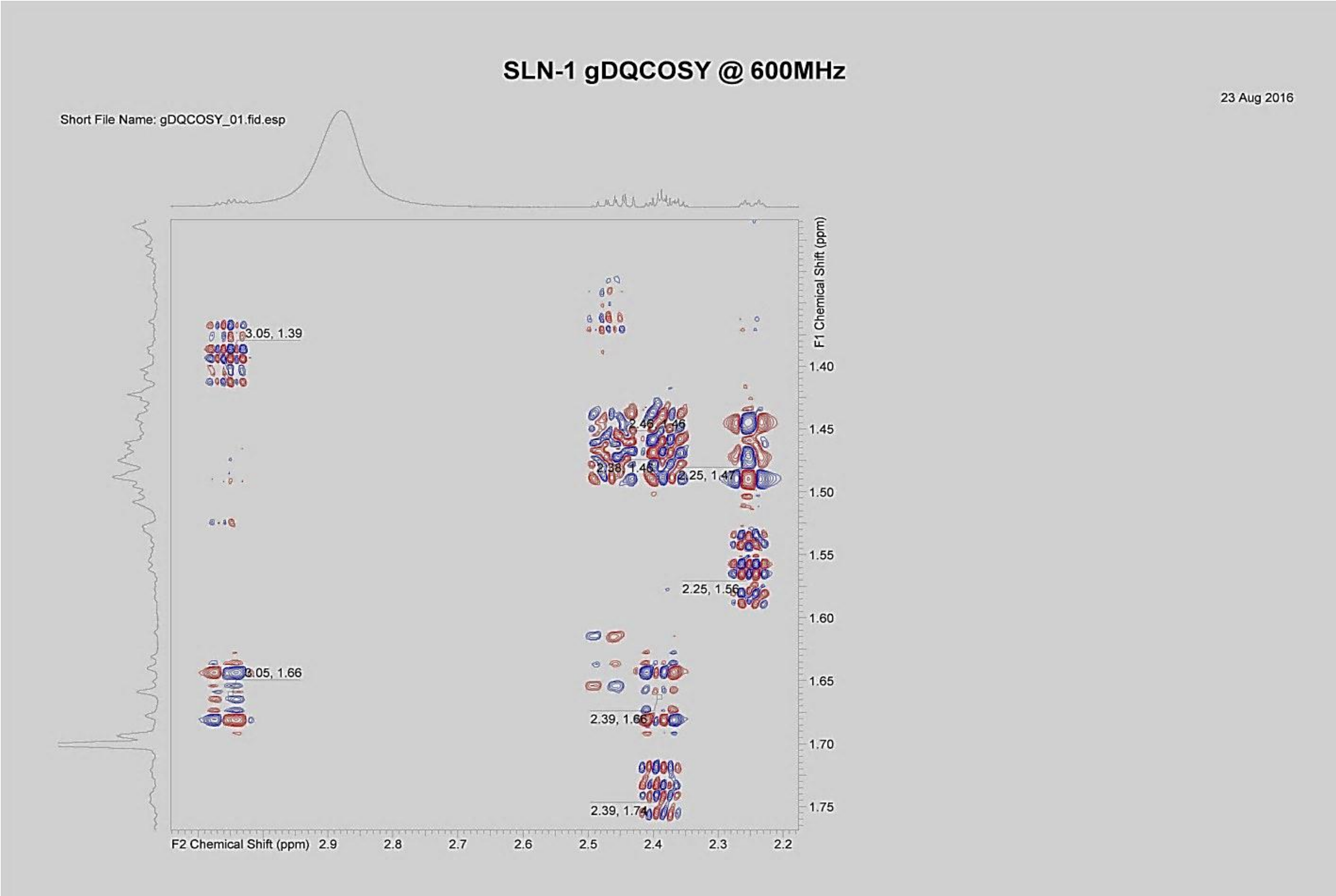

SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHSQCAD_PS_01.fid.esp
3.05, 48.03
1.66, 50.00
1.49, 50.75
1.42, 55.61
F1 Chemical Shift (ppm)
50.0
50.5
51.0
51.5
52.0
52.5
53.0
53.5
54.0
54.5
55.0
55.5
56.0
56.5
57.0
F2 Chemical Shift (ppm)
2.5
2.0
1.5


SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHSQCAD_PS_01.fid.esp
4.72, 110.09
4.59, 110.09
F1 Chemical Shift (ppm)
109.5
109.6
109.7
109.8
109.9
110.0
110.1
110.2
110.3
110.4
110.5
110.6
110.7
110.8
110.9
F2 Chemical Shift (ppm)
4.65
4.60

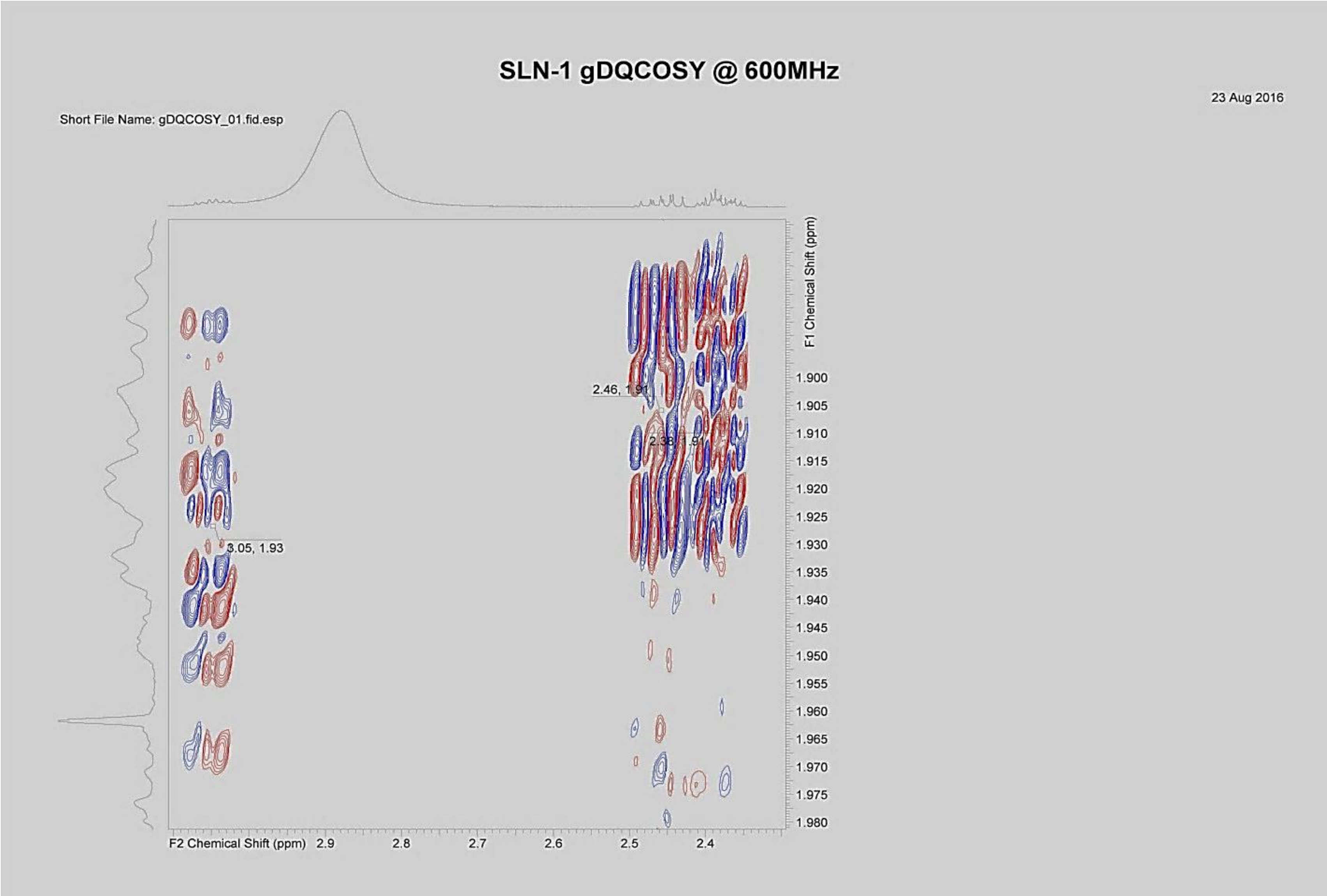

SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHSQCAD_PS_01.fid.esp
4.72, 110.09
4.59, 110.09
F1 Chemical Shift (ppm)
109.5
109.6
109.7
109.8
109.9
110.0
110.1
110.2
110.3
110.4
110.5
110.6
110.7
110.8
110.9
F2 Chemical Shift (ppm)
4.65
4.60

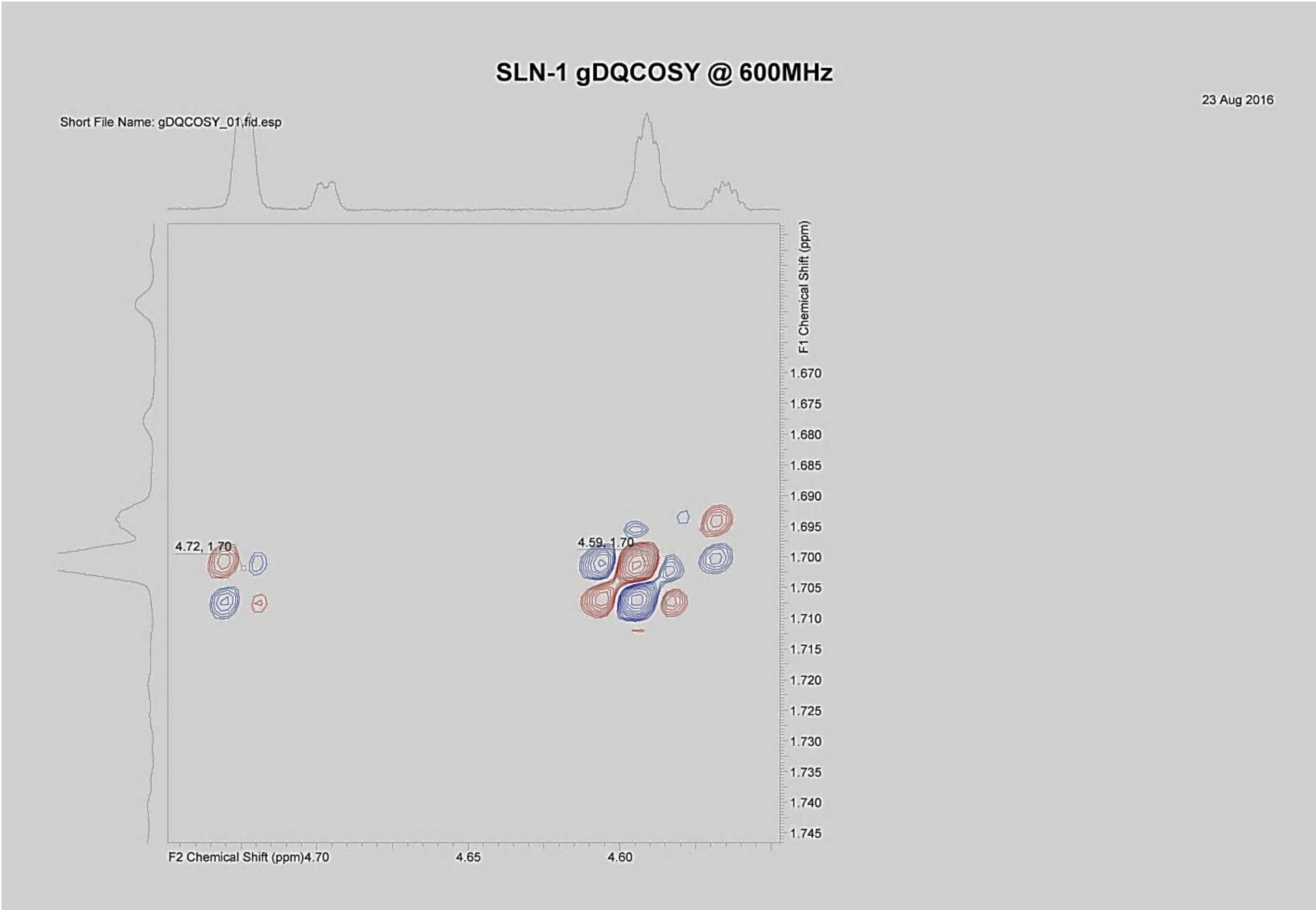

SLN-1 gDQCOSY @ 600MHz
23 Aug 2016
Gradient pulse duration 0.001
Gradient Strength1 473
Short File Name: gDQCOSY_01.fid.esp
1.73, 1.10
2.25, 1.21
1.73, 1.30
3.05, 1.39
2.25, 1.47
1.48, 1.21
1.48, 1.30
3.05, 1.66
2.46, 1.46
2.38, 1.46
1.90, 1.46
1.93, 1.50
2.46, 1.91
2.39, 1.66
4.59, 1.70
2.39, 1.74
2.38, 1.91
3.05, 1.93
2.46, 2.38
4.72, 4.59
F2 Chemical Shift (ppm)
F1 Chemical Shift (ppm)

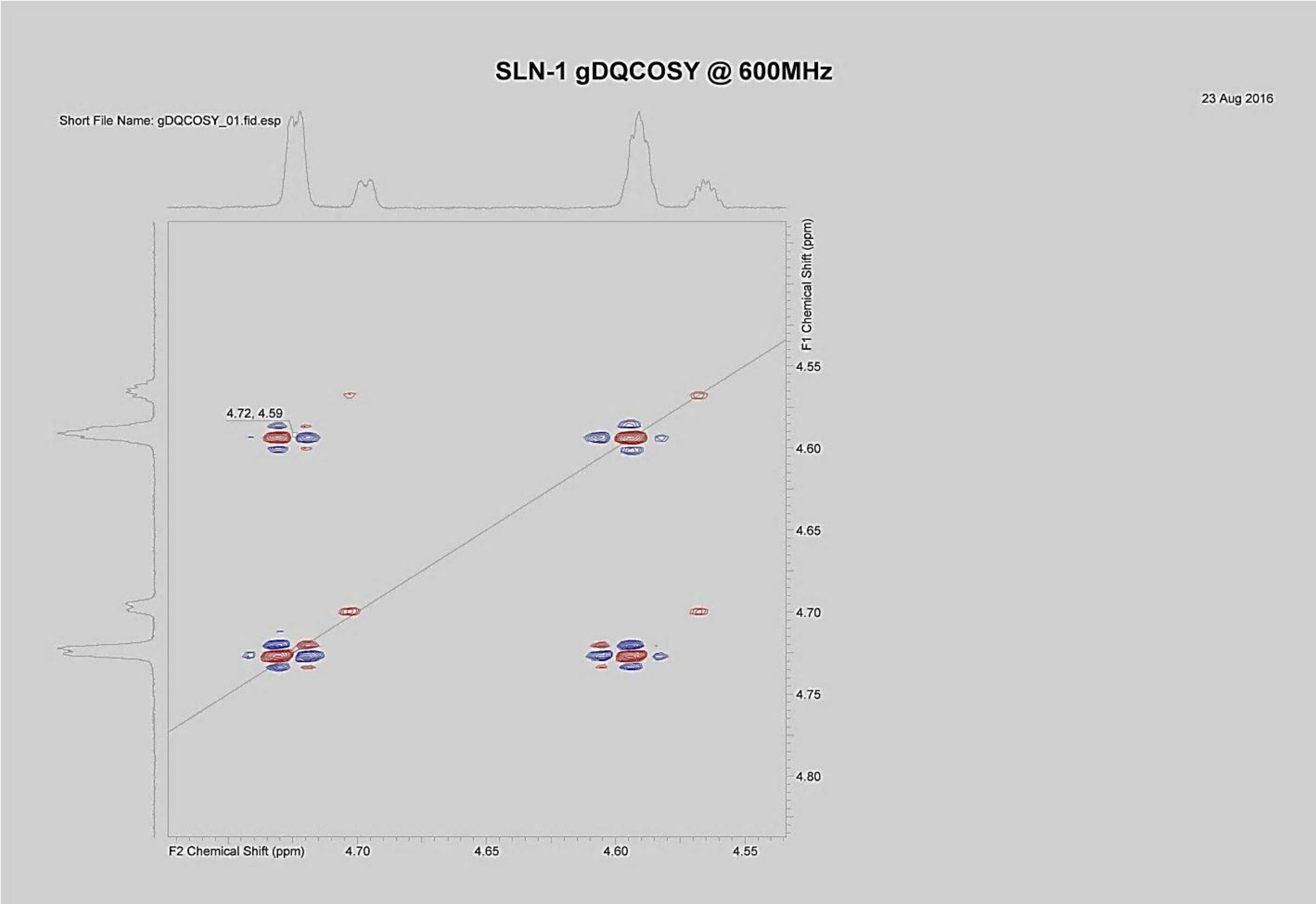

SLN-1 gDQCOSY @ 600MHz
23 Aug 2016
Short File Name: gDQCOSY_01.fid.esp
F1 Chemical Shift (ppm)
F2 Chemical Shift (ppm)
2.39, 1.09
1.73, 1.10
1.47, 1.11
1.56, 1.21
1.48, 1.21
2.25, 1.21
1.73, 1.30
1.48, 1.30



SLN-1 gDQCOSY @ 600MHz
23 Aug 2016
Short File Name: gDQCOSY_01.fid.esp
3.05, 1.39
3.05, 1.66
2.46, 1.46
2.38, 1.48
2.25, 1.47
2.25, 1.56
2.39, 1.66
2.39, 1.74
F1 Chemical Shift (ppm)
1.40
1.45
1.50
1.55
1.60
1.65
1.70
1.75
F2 Chemical Shift (ppm)
2.9
2.8
2.7
2.6
2.5
2.4
2.3
2.2


SLN-1 gDQCOSY @ 600MHz
23 Aug 2016
Short File Name: gDQCOSY_01.fid.esp
2.46, 1.91
2.38, 1.91
3.05, 1.93
F1 Chemical Shift (ppm)
1.900
1.905
1.910
1.915
1.920
1.925
1.930
1.935
1.940
1.945
1.950
1.955
1.960
1.965
1.970
1.975
1.980
F2 Chemical Shift (ppm) 2.9
2.8
2.7
2.6
2.5
2.4


SLN-1 gDQCOSY @ 600MHz
23 Aug 2016
Short File Name: gDQCOSY_01.fid.esp
4.72, 1.70
4.59, 1.70
F1 Chemical Shift (ppm)
1.670
1.675
1.680
1.685
1.690
1.695
1.700
1.705
1.710
1.715
1.720
1.725
1.730
1.735
1.740
1.745
F2 Chemical Shift (ppm)4.70
4.65
4.60

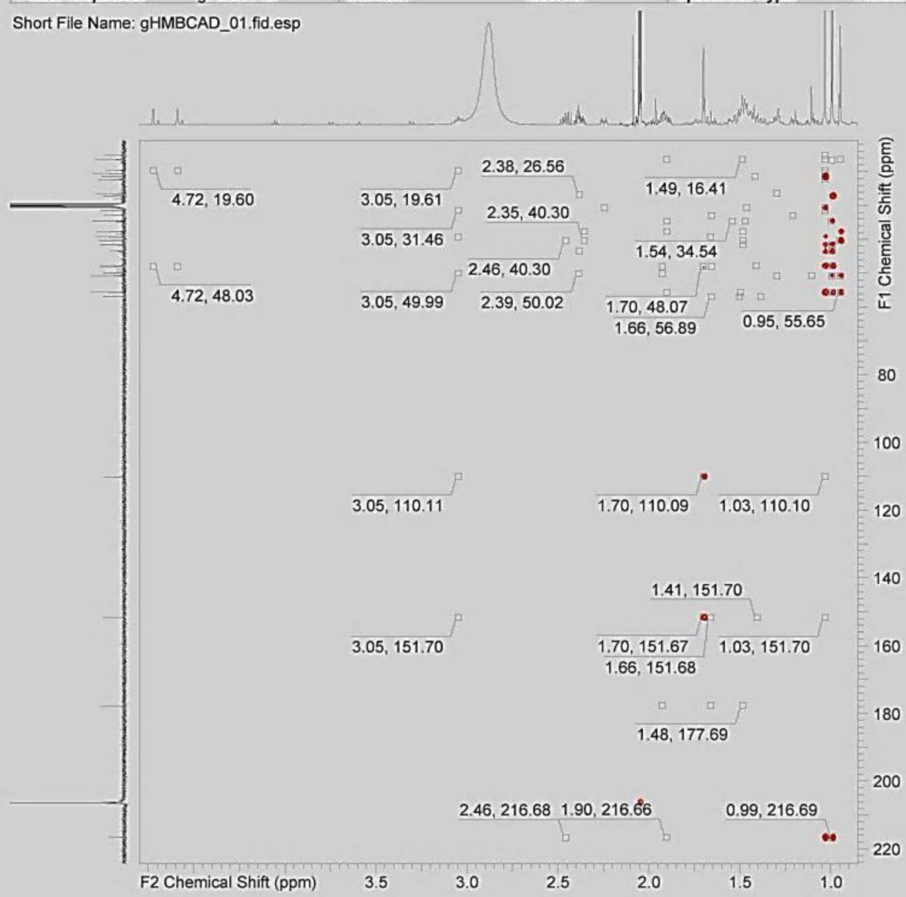

SLN-1 gDQCOSY @ 600MHz
23 Aug 2016
Short File Name: gDQCOSY_01.fid.esp
4.72, 4.59
F1 Chemical Shift (ppm)
4.55
4.60
4.65
4.70
4.75
4.80
F2 Chemical Shift (ppm)
4.70
4.65
4.60
4.55

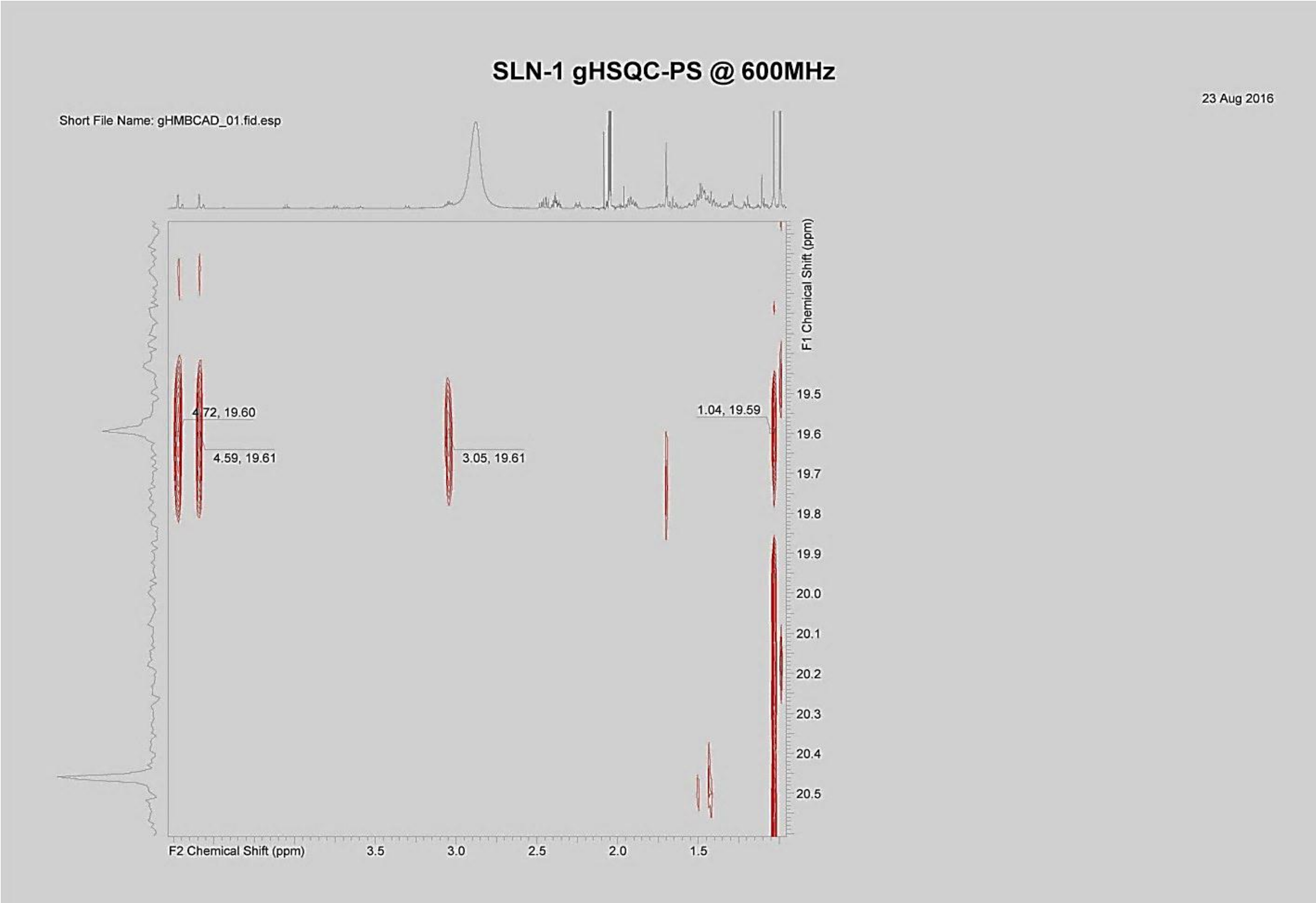
SLN-1 gHSQC-PS @ 600MHz

23 Aug 2016

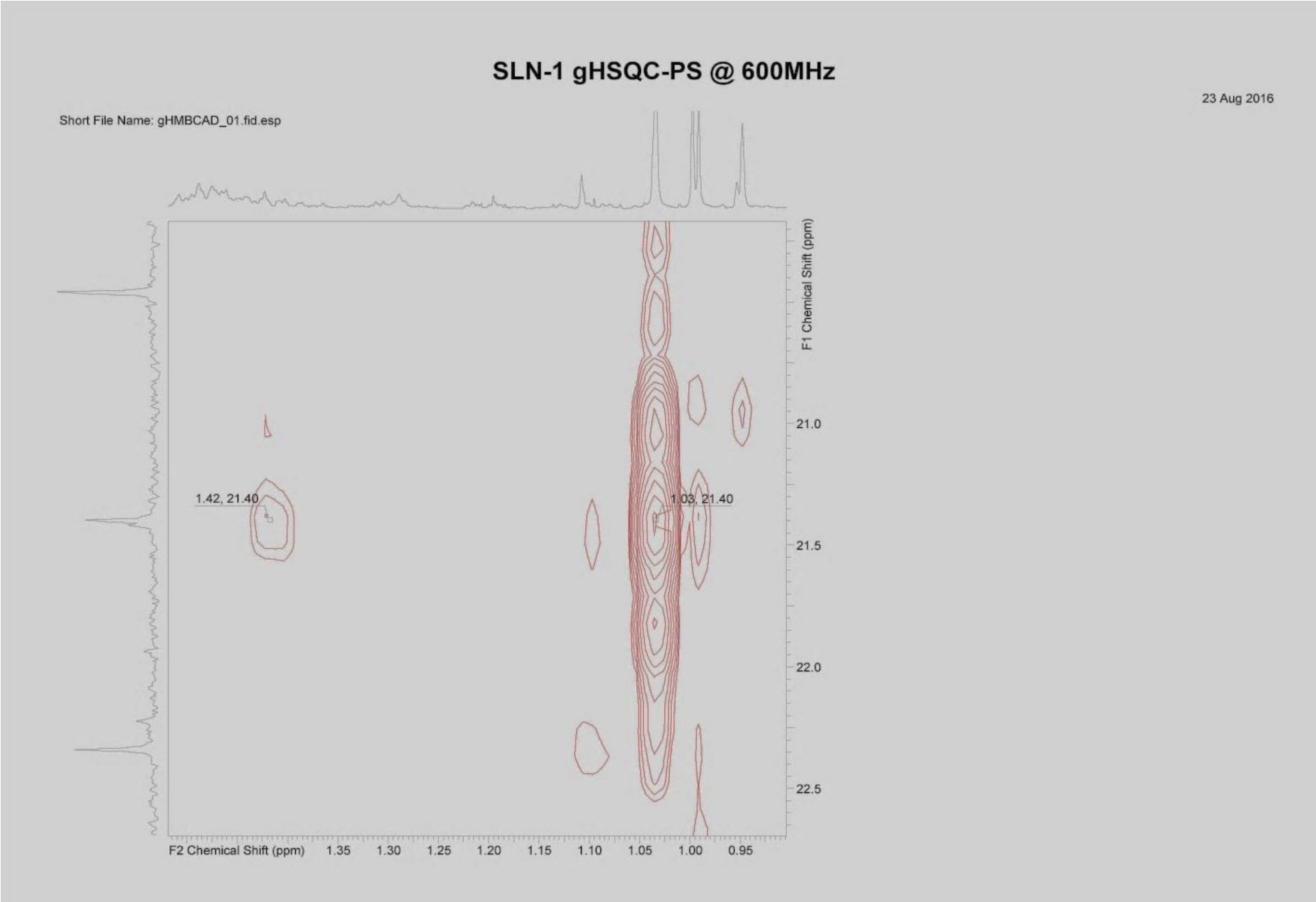
Acquisition Time (sec)	(0.1500, 0.0151)	***Date***	29 Mar 2016 11:04:26			***Date Stamp***	Mar 22 2016
File Name	G:\SLN\SNL-1_20160322_01\gHMBCAD_01.fid\fid			***Frequency (MHz)***	(599.7388, 150.8044)		
Nucleus	(1H, 13C)	***Number of Transients***	16	***Original Points Count***	(404, 512)	***Points Count***	(512, 2048)
Pulse Sequence	gHMBCAD	***Solvent***	acetone	***Spectrum Type***	HMBC	***Sweep Width (Hz)***	(2688.70, 33910.49)

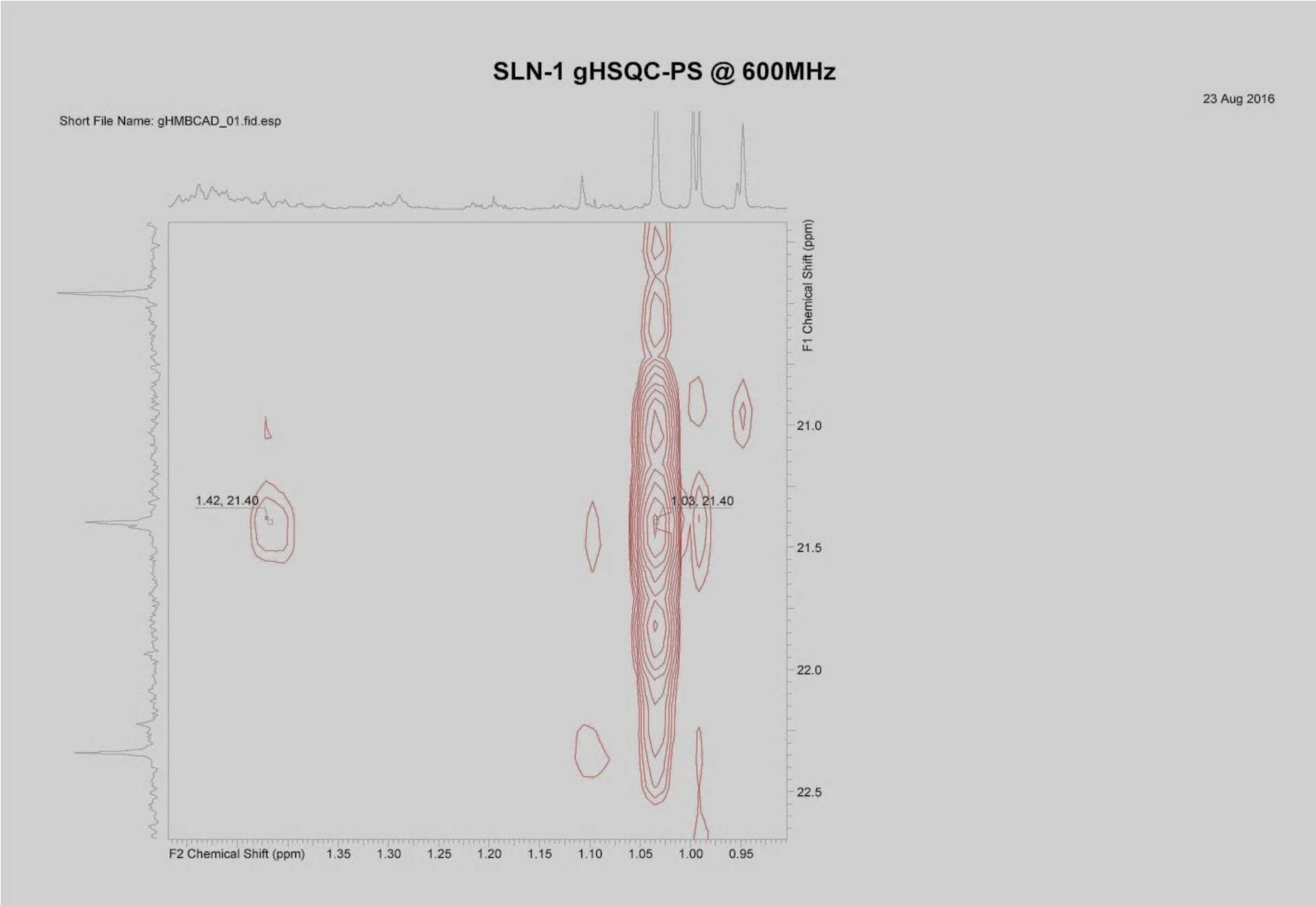
Short File Name: gHMBCAD_01.fid.esp



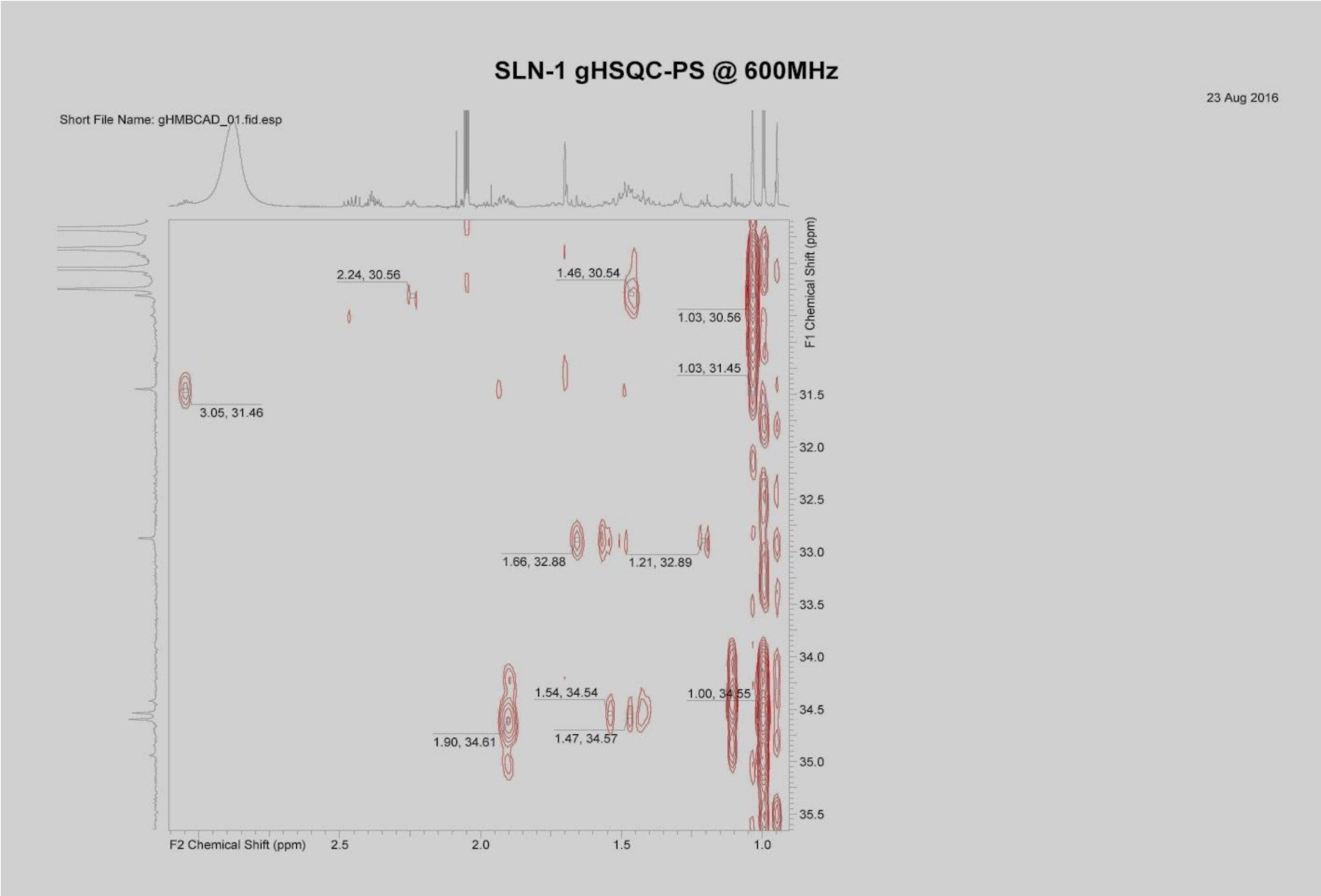


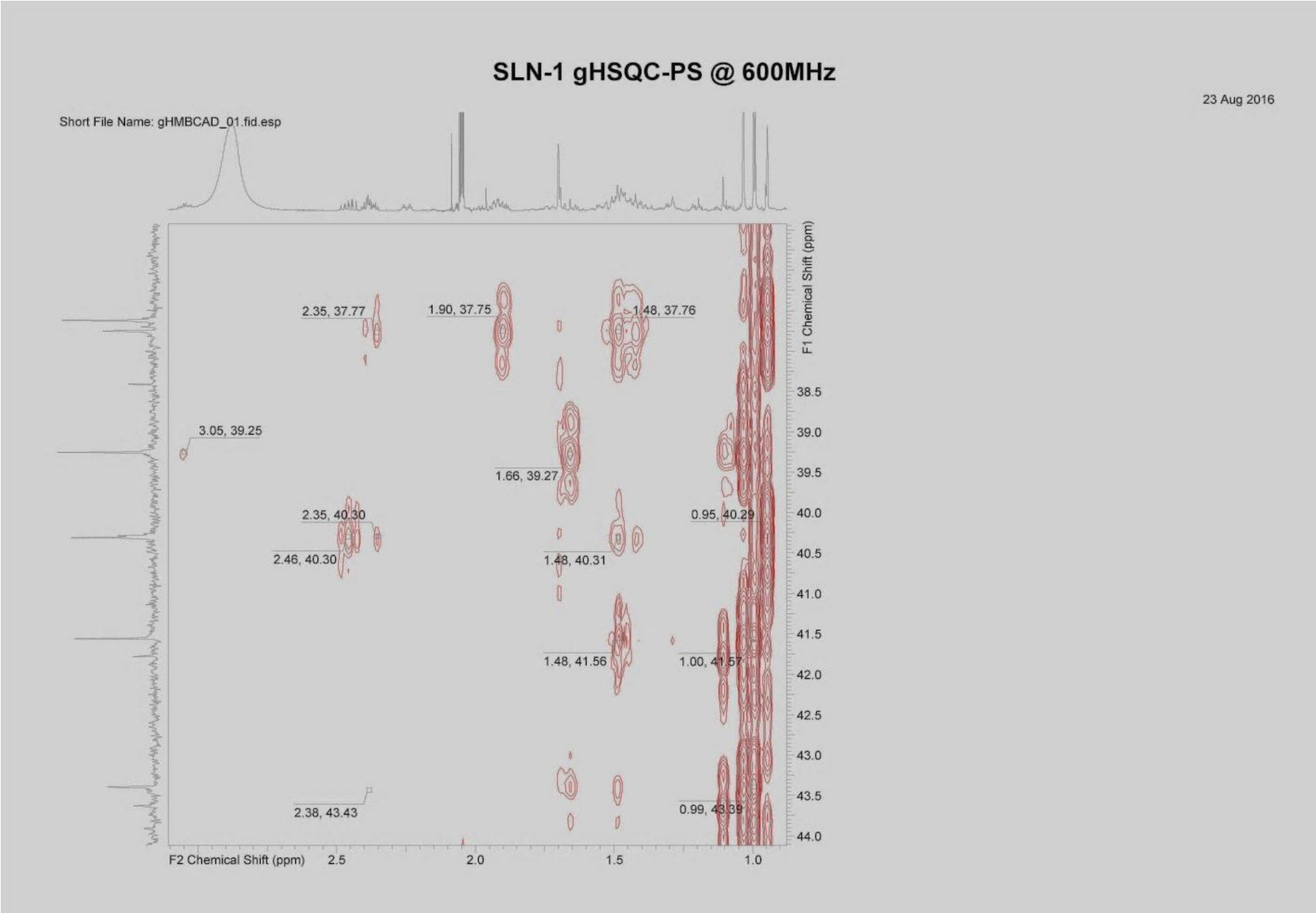
SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
4.72, 19.60
4.59, 19.61
3.05, 19.61
1.04, 19.59
F1 Chemical Shift (ppm)
19.5
19.6
19.7
19.8
19.9
20.0
20.1
20.2
20.3
20.4
20.5
F2 Chemical Shift (ppm)
3.5
3.0
2.5
2.0
1.5


SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
1.42, 21.40
1.03, 21.40
F1 Chemical Shift (ppm)
21.0
21.5
22.0
22.5
F2 Chemical Shift (ppm)
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95

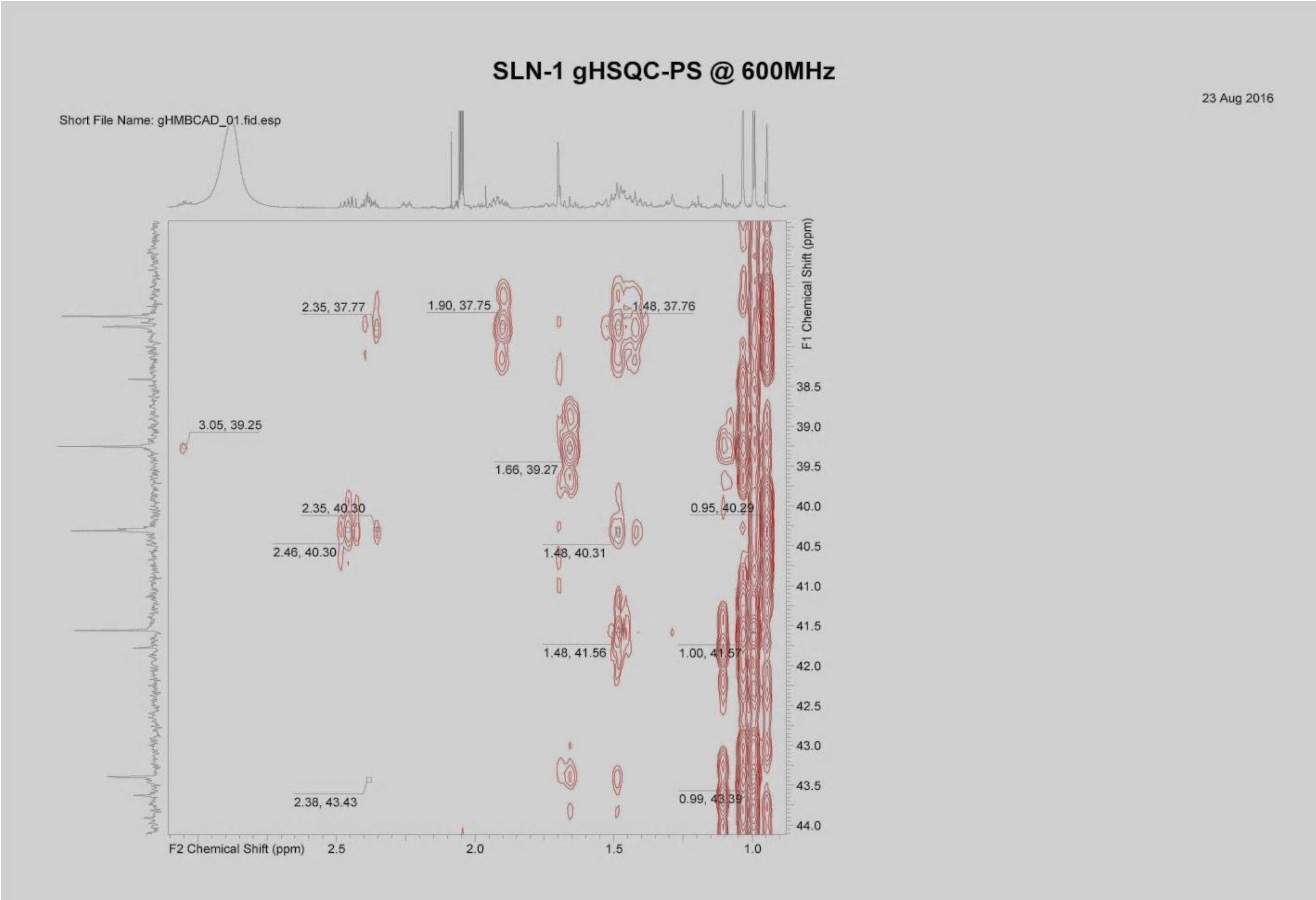


SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
1.42, 21.40
1.03, 21.40
F1 Chemical Shift (ppm)
21.0
21.5
22.0
22.5
F2 Chemical Shift (ppm)
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95

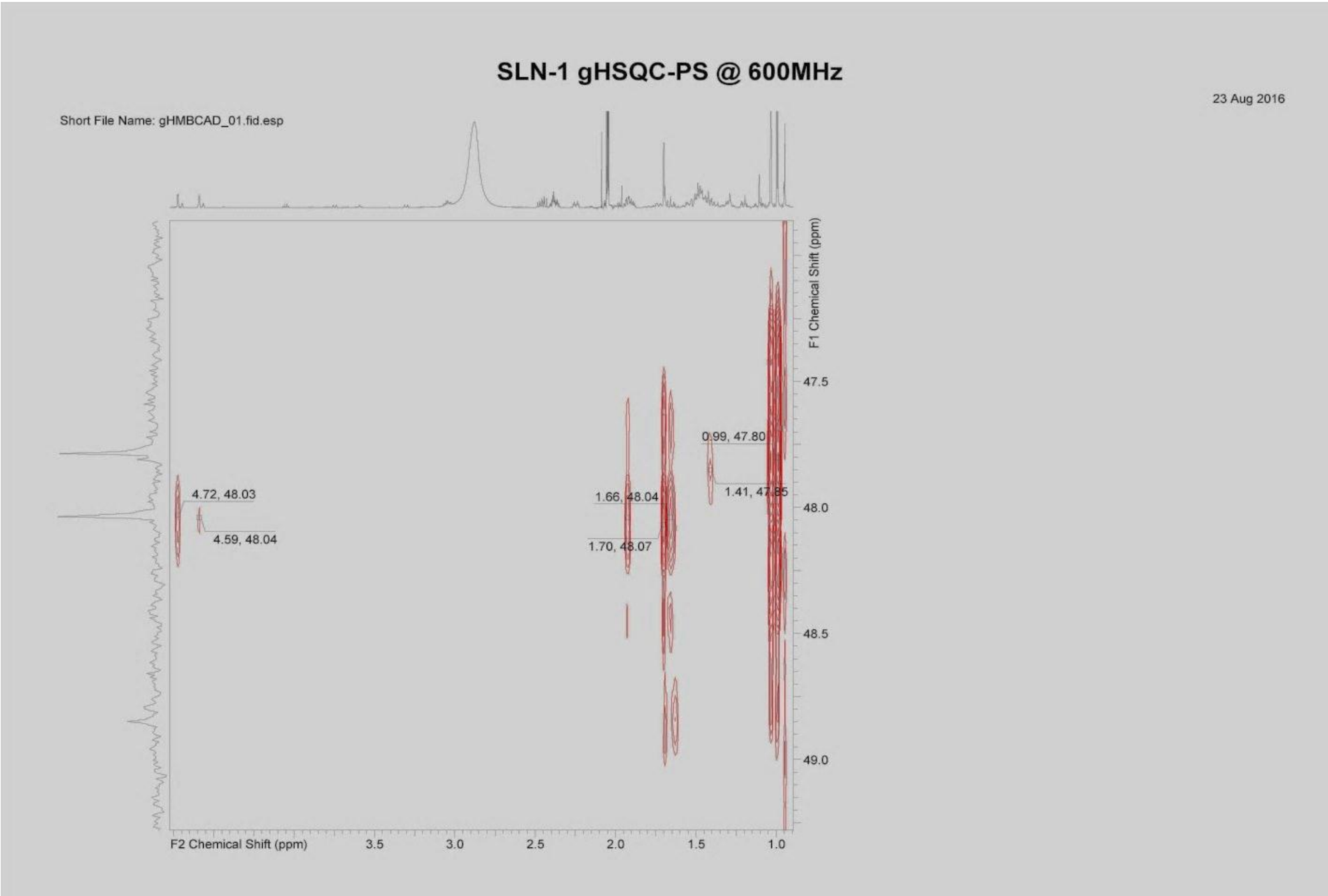

SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
2.24, 30.56
1.46, 30.54
1.03, 30.56
1.03, 31.45
3.05, 31.46
1.66, 32.88
1.21, 32.89
1.54, 34.54
1.00, 34.55
1.90, 34.61
1.47, 34.57
F1 Chemical Shift (ppm)
F2 Chemical Shift (ppm)



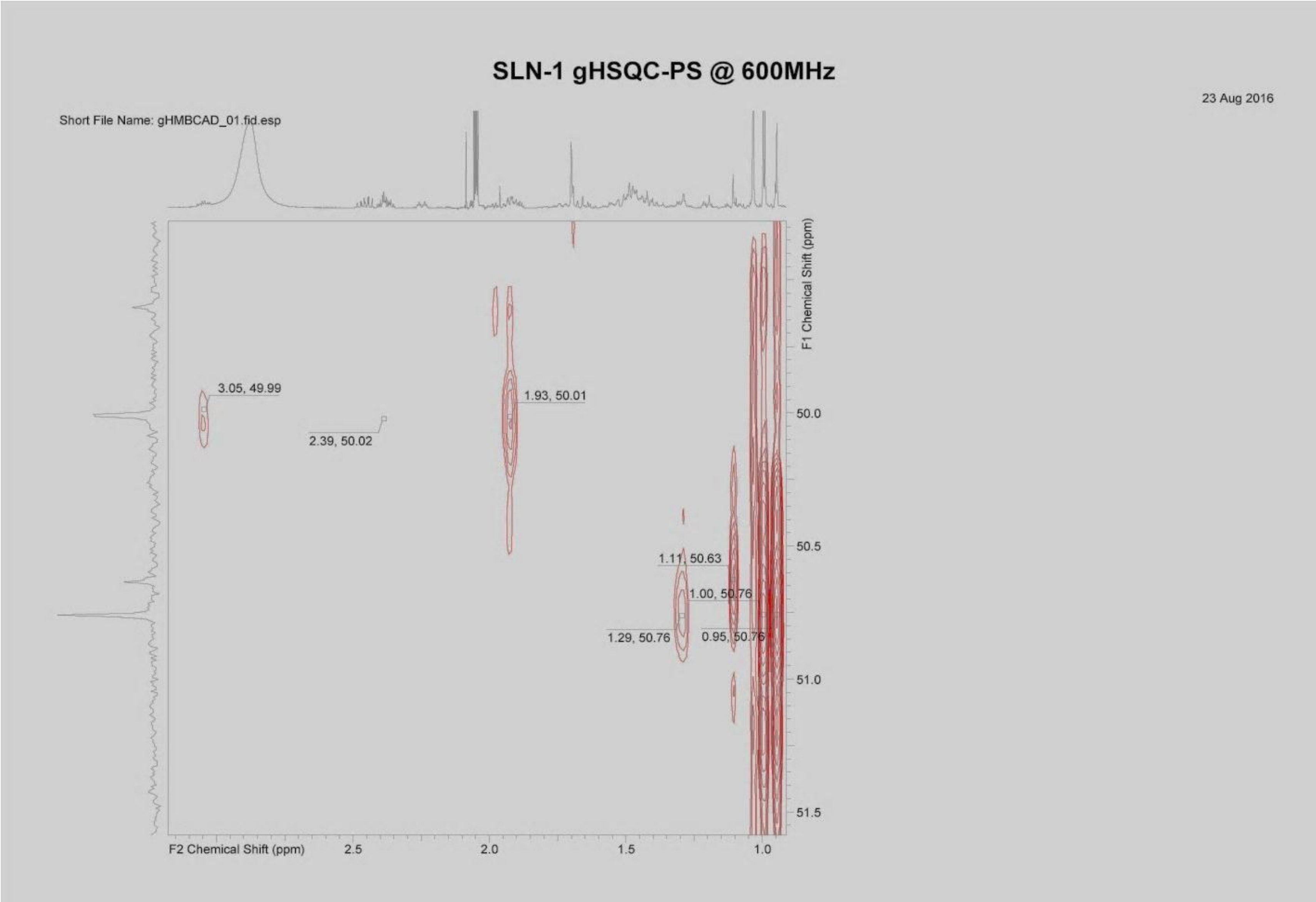
SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
2.35, 37.77
1.90, 37.75
1.48, 37.76
3.05, 39.25
1.66, 39.27
2.35, 40.30
0.95, 40.29
2.46, 40.30
1.48, 40.31
1.48, 41.56
1.00, 41.57
2.38, 43.43
0.99, 43.39
F1 Chemical Shift (ppm)
F2 Chemical Shift (ppm)

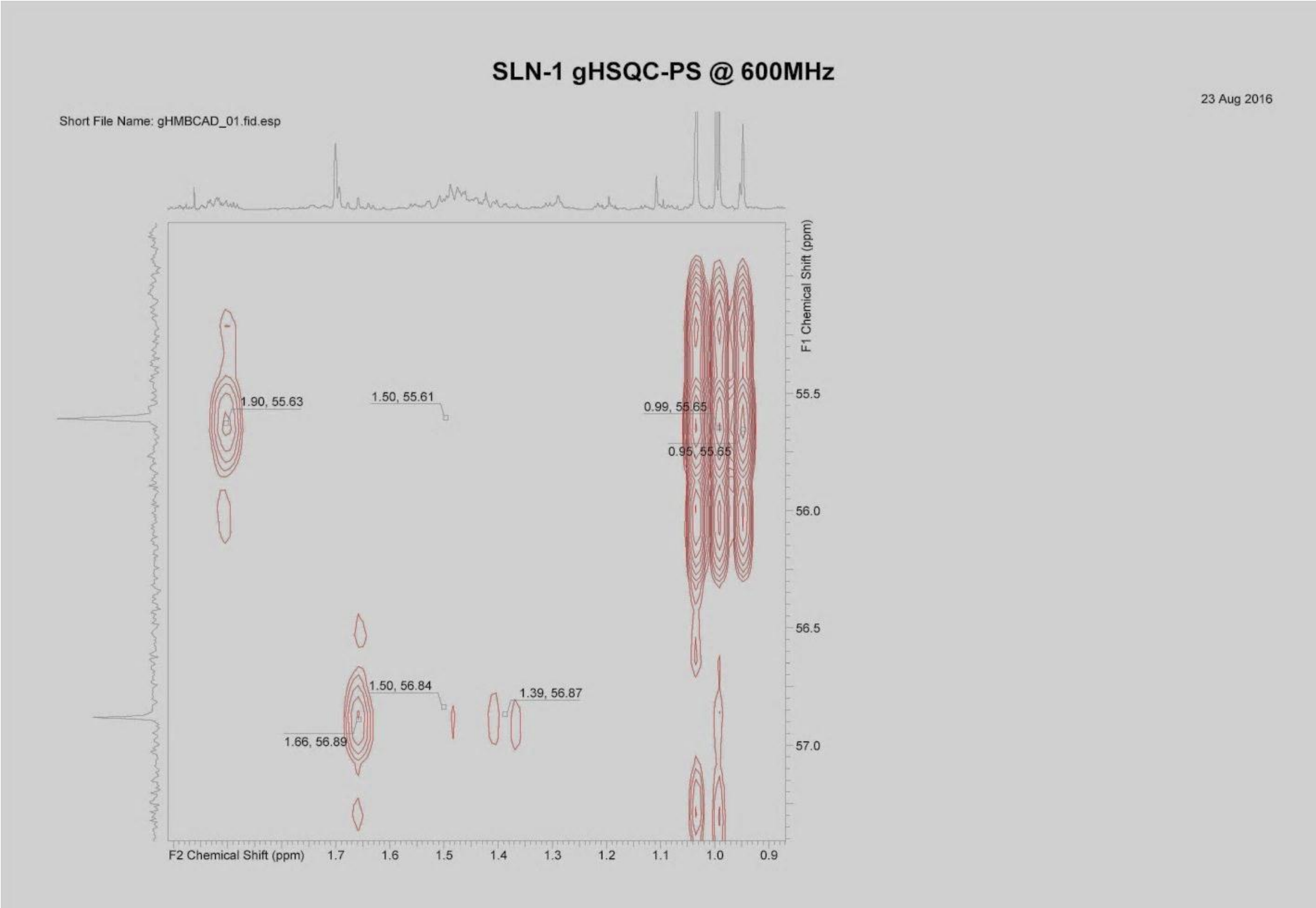


SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
2.35, 37.77
1.90, 37.75
1.48, 37.76
3.05, 39.25
1.66, 39.27
2.35, 40.30
2.46, 40.30
1.48, 40.31
0.95, 40.29
1.48, 41.56
1.00, 41.57
2.38, 43.43
0.99, 43.39
F2 Chemical Shift (ppm)
F1 Chemical Shift (ppm)

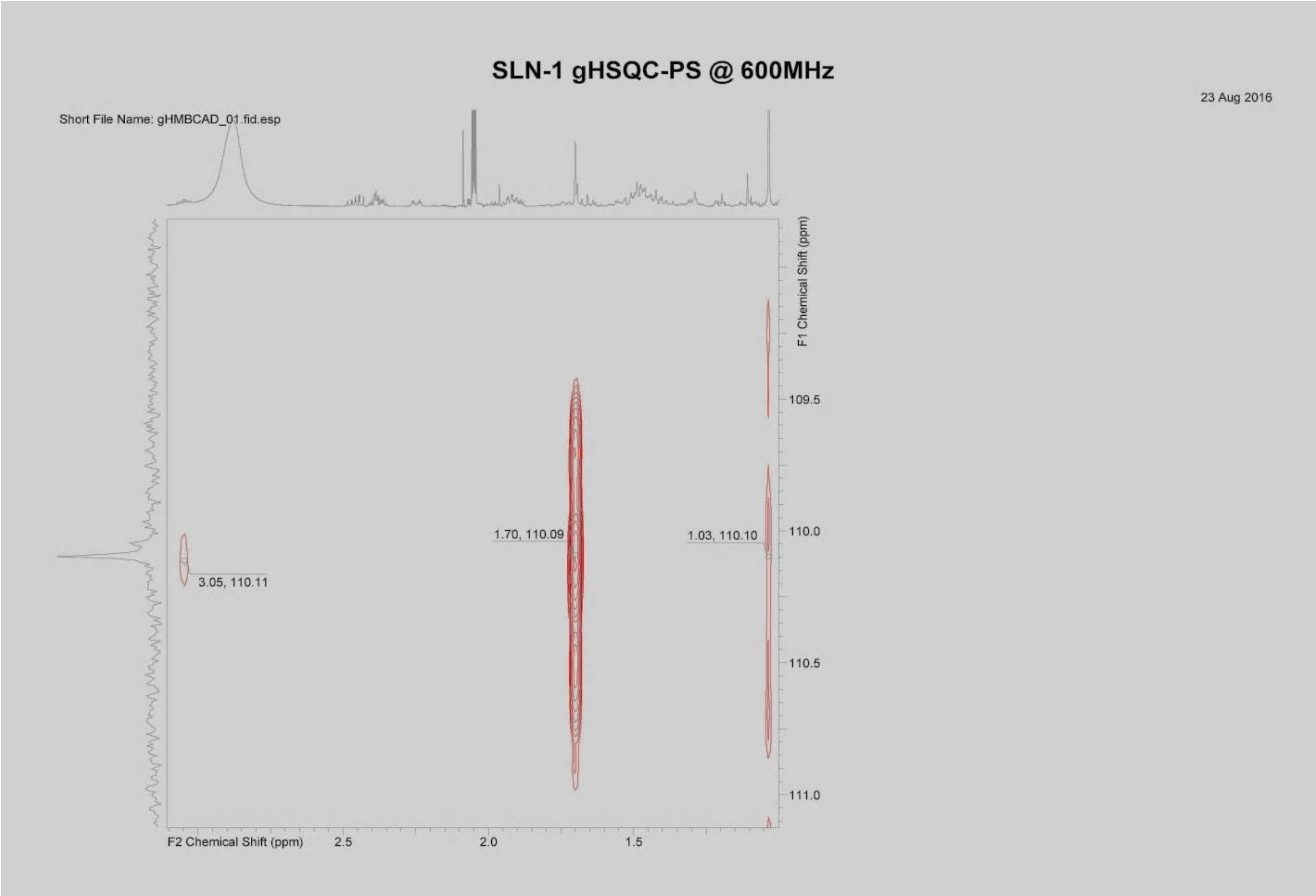


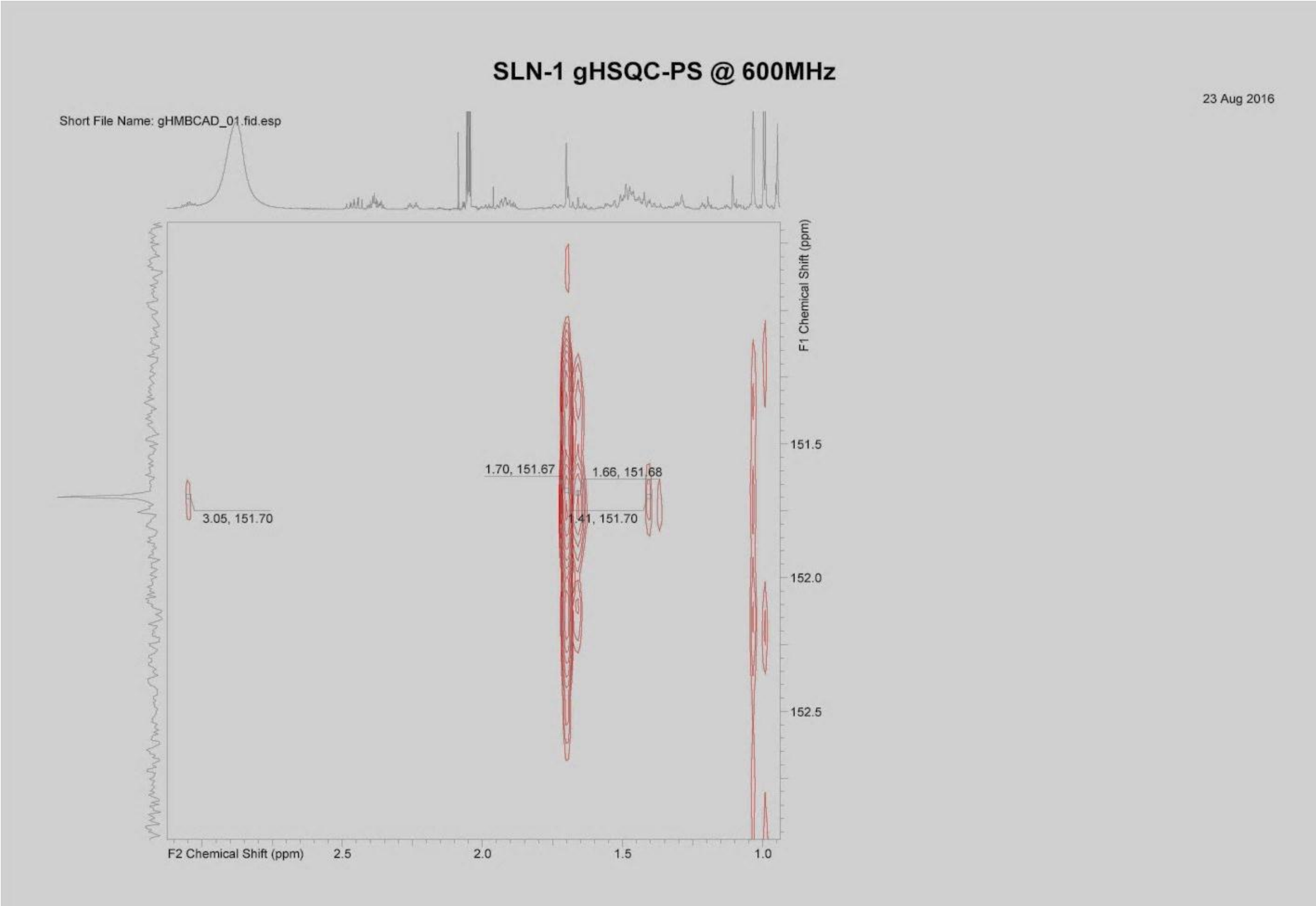
SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
4.72, 48.03
4.59, 48.04
1.66, 48.04
1.70, 48.07
0.99, 47.80
1.41, 47.85
F1 Chemical Shift (ppm)
47.5
48.0
48.5
49.0
F2 Chemical Shift (ppm)
3.5
3.0
2.5
2.0
1.5
1.0

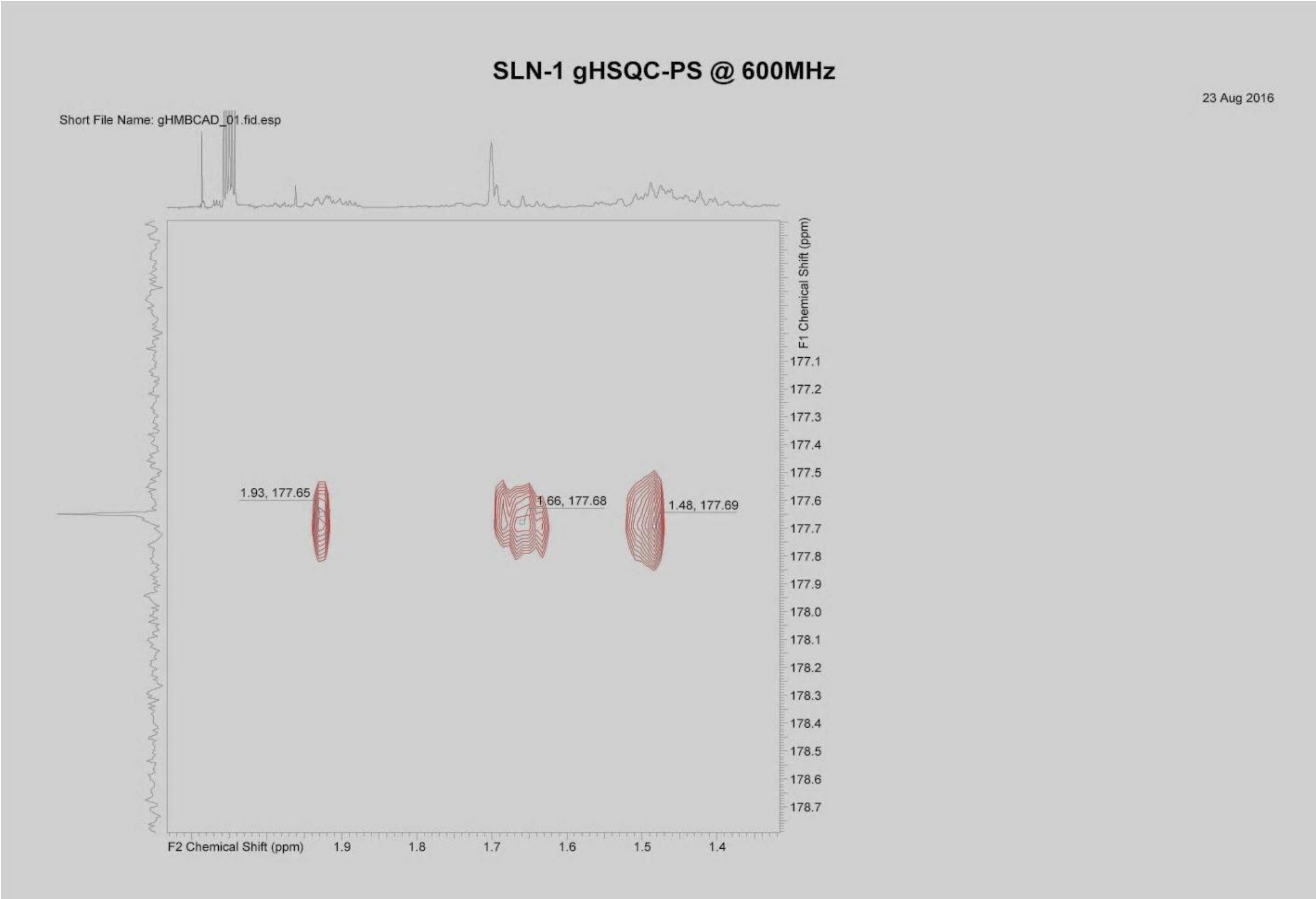

SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
3.05, 49.99
2.39, 50.02
1.93, 50.01
1.11, 50.63
1.00, 50.76
1.29, 50.76
0.95, 50.76
F1 Chemical Shift (ppm)
50.0
50.5
51.0
51.5
F2 Chemical Shift (ppm)
2.5
2.0
1.5
1.0



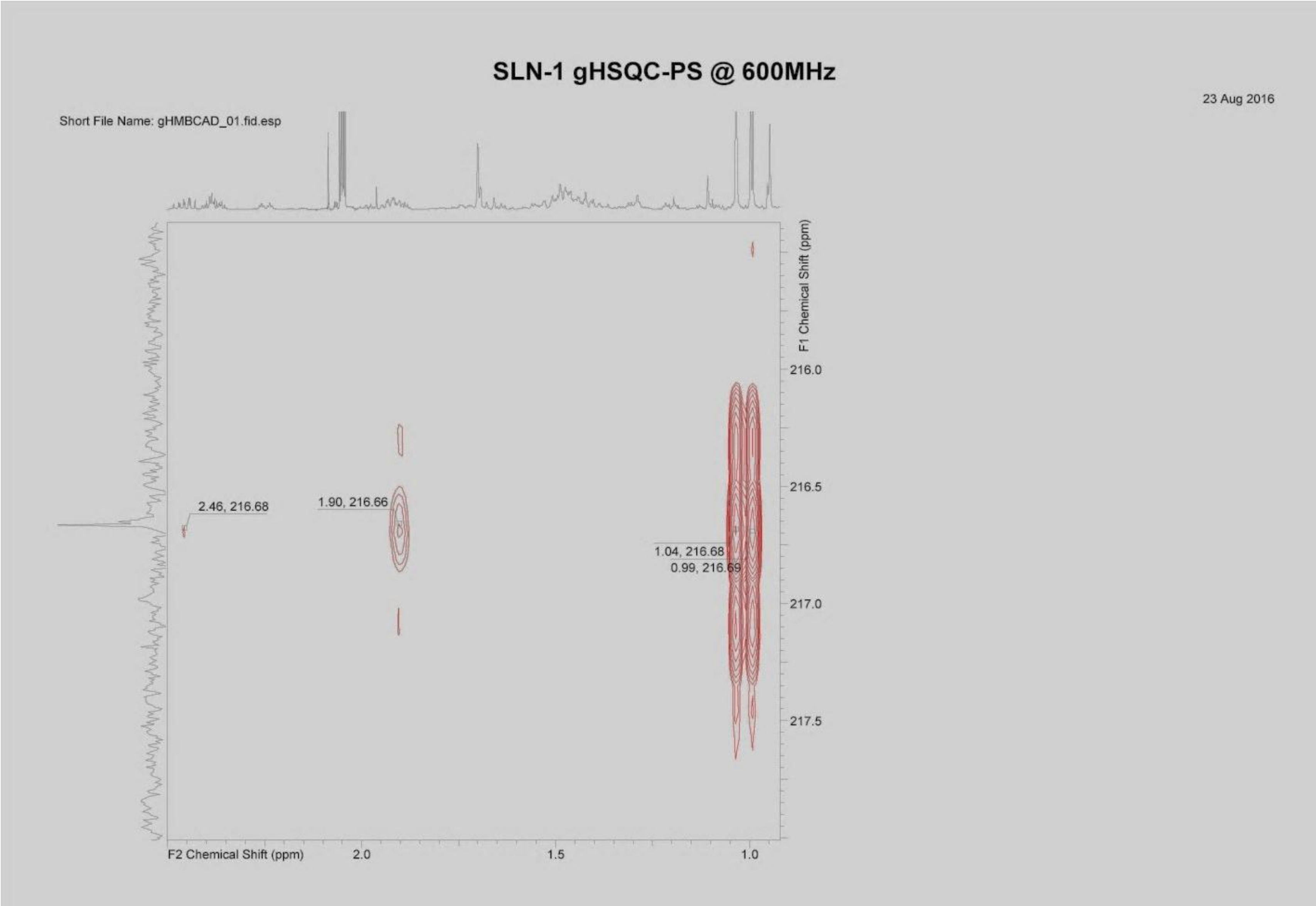
SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
1.90, 55.63
1.50, 55.61
0.99, 55.65
0.95, 55.65
1.50, 56.84
1.39, 56.87
1.66, 56.89
F1 Chemical Shift (ppm)
55.5
56.0
56.5
57.0
F2 Chemical Shift (ppm)
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9


SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
3.05, 110.11
1.70, 110.09
1.03, 110.10
F1 Chemical Shift (ppm)
109.5
110.0
110.5
111.0
F2 Chemical Shift (ppm)
2.5
2.0
1.5


SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
3.05, 151.70
1.70, 151.67
1.66, 151.68
1.41, 151.70
F1 Chemical Shift (ppm)
151.5
152.0
152.5
F2 Chemical Shift (ppm)
2.5
2.0
1.5
1.0

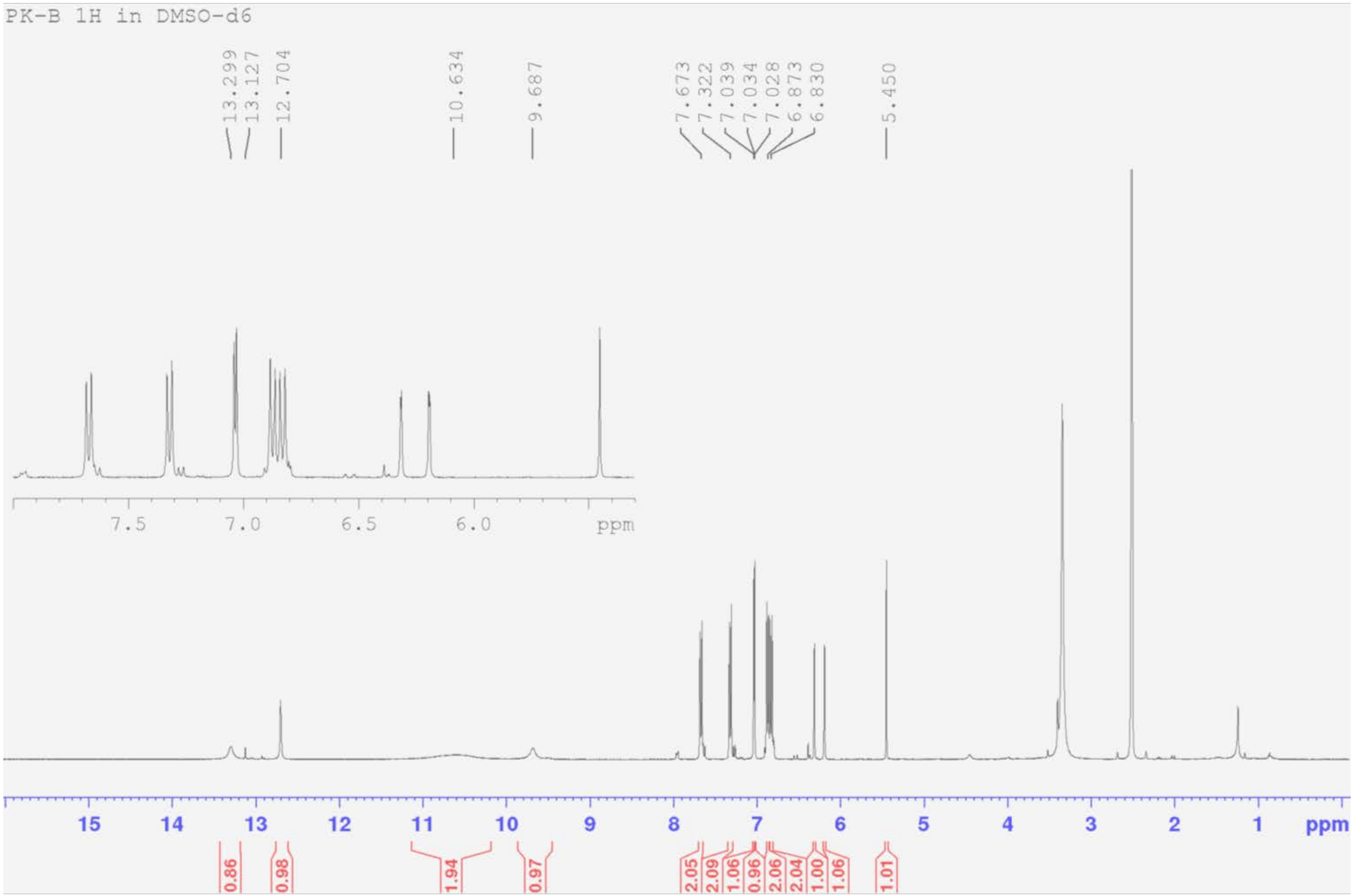


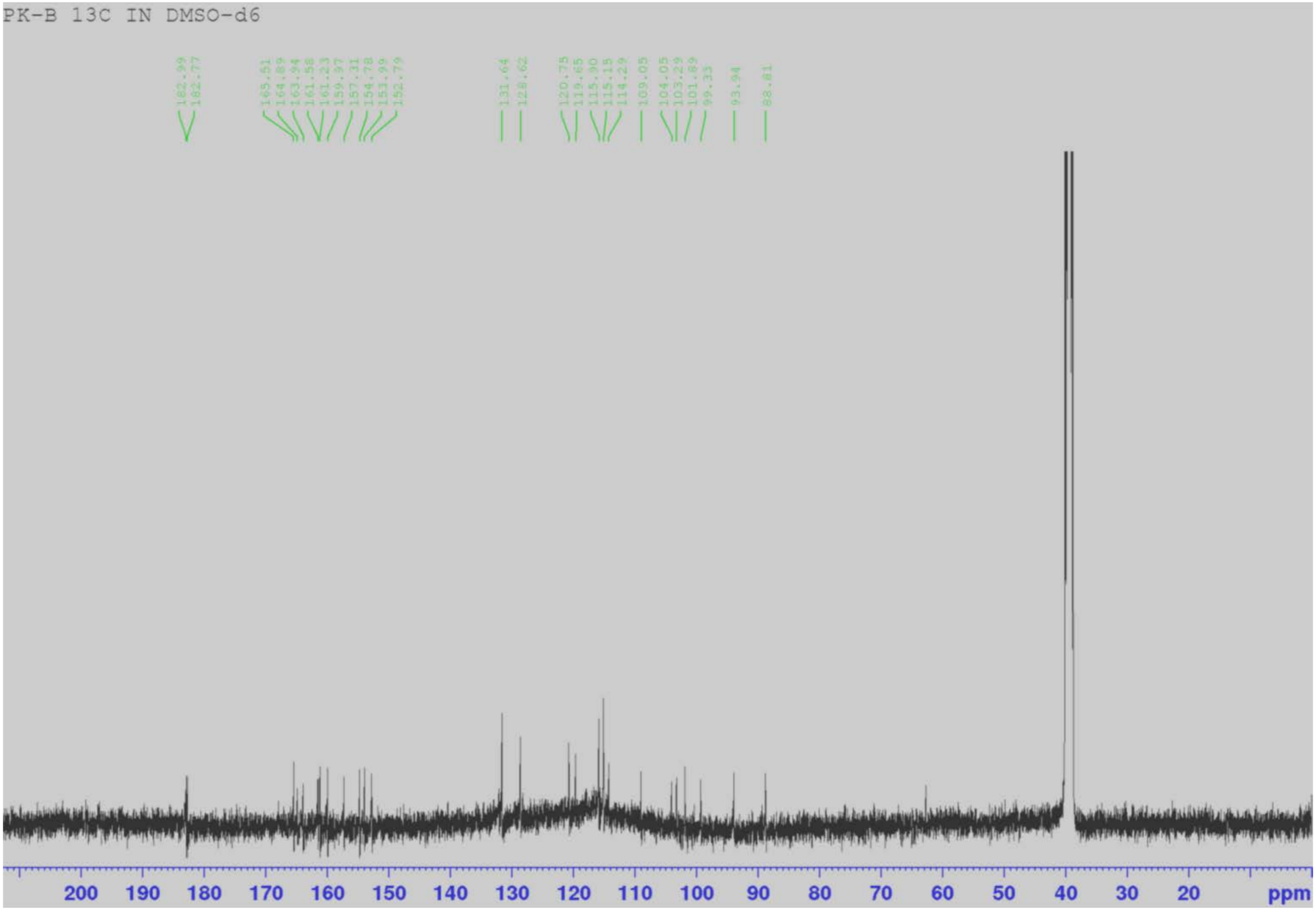
SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
1.93, 177.65
1.66, 177.68
1.48, 177.69
F1 Chemical Shift (ppm)
F2 Chemical Shift (ppm)

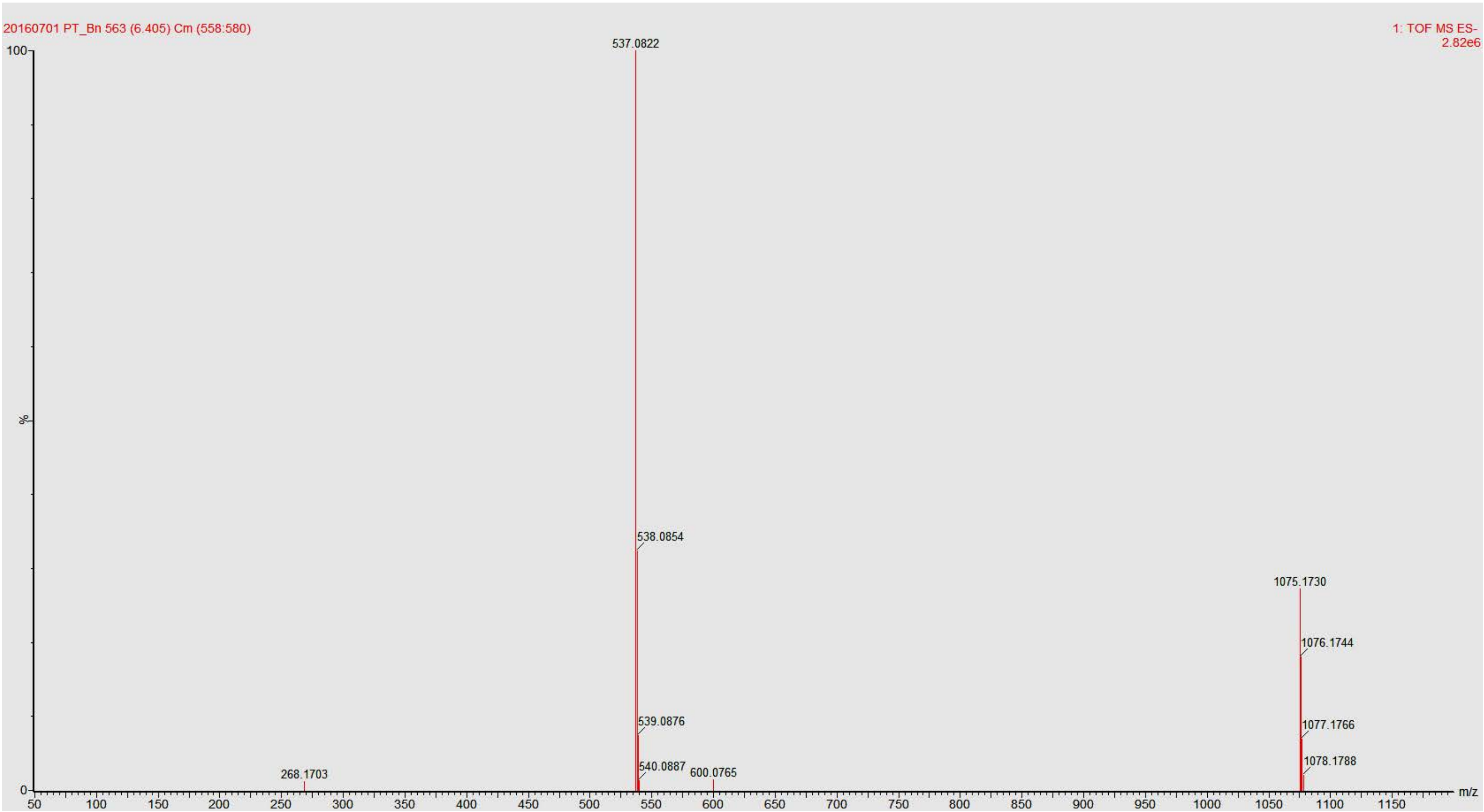


SLN-1 gHSQC-PS @ 600MHz
23 Aug 2016
Short File Name: gHMBCAD_01.fid.esp
2.46, 216.68
1.90, 216.66
1.04, 216.68
0.99, 216.69
F1 Chemical Shift (ppm)
216.0
216.5
217.0
217.5
F2 Chemical Shift (ppm)
2.0
1.5
1.0

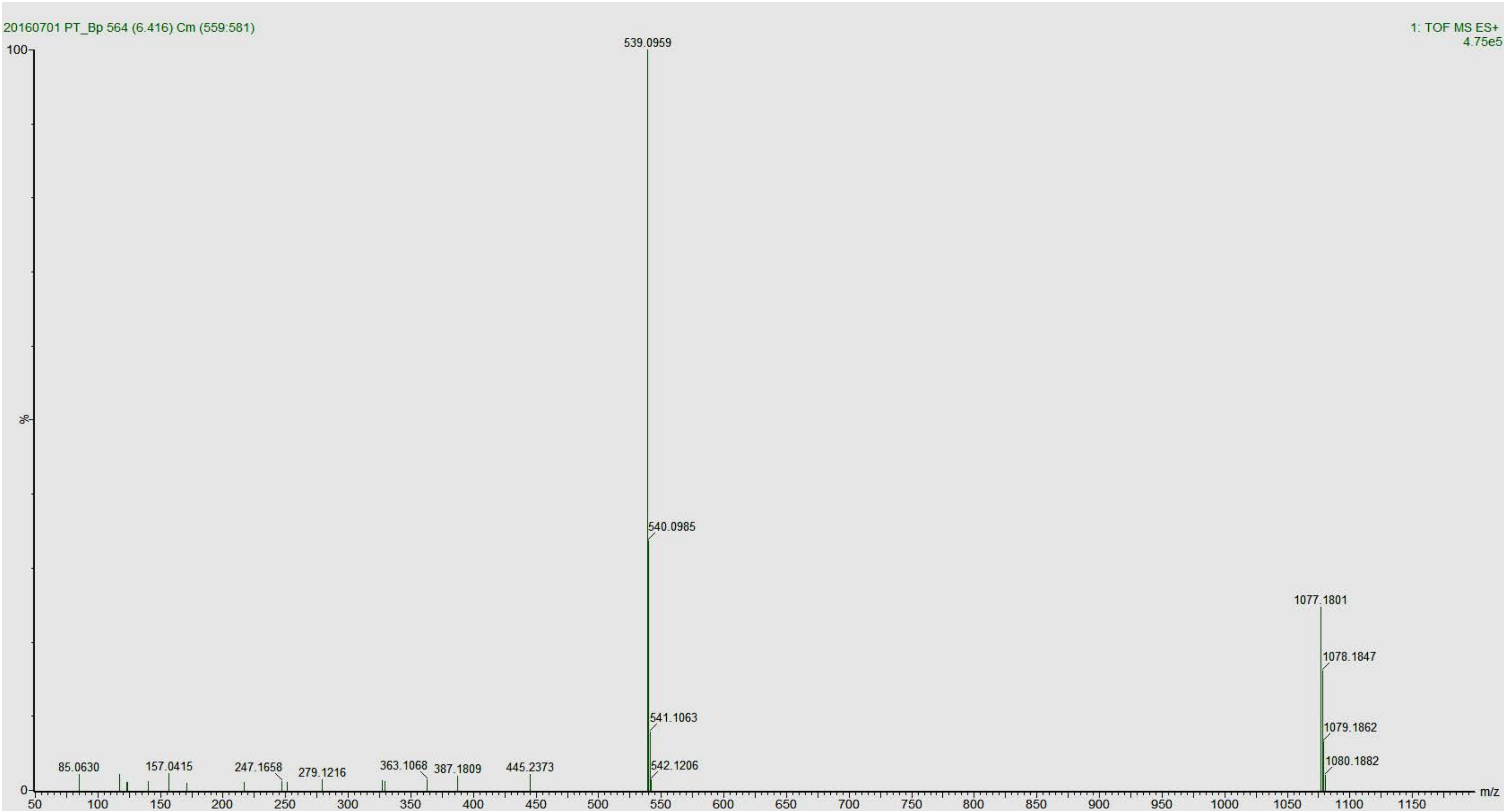
Appendix B: Structure elucidation of compound PK-B




PK-B 13C IN DMSO-d6
182.99
182.77
165.51
164.89
163.94
161.58
161.23
159.97
157.31
154.78
153.99
152.79
131.64
128.62
120.75
119.65
115.90
115.15
114.29
109.05
104.05
103.29
101.89
99.33
93.94
88.81
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm



$[M-H]^-$ = 537.0822 for C30H17O10



[M-H]$^+$ = 539.0959 for C30H19O10

Appendix C: Published articles

PHYTOTHERAPY RESEARCH
Phytother. Res. (2016)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.5706

Antimycobacterial Activity and Low Cytotoxicity of Leaf Extracts of Some African Anacardiaceae Tree Species

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[2]Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa
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Treatment of tuberculosis (TB) is a challenge because of multidrug-resistant and extremely drug-resistant strains of *Mycobacterium tuberculosis.* Plant species contain antimicrobial compounds that may lead to new anti-TB drugs. Previous screening of some tree species from the Anacardiaceae family revealed the presence of antimicrobial activity, justifying further investigations. Leaf extracts of 15 Anacardiaceae tree species were screened for antimycobacterial activity using a twofold serial microdilution assay against the pathogenic *Mycobacterium bovis* and multidrug resistant *M. tuberculosis* and rapidly growing mycobacteria, *Mycobacterium smegmatis, Mycobacterium fortuitum* and *Mycobacterium aurum*. The vaccine strain, *M. bovis* and an avirulent strain, H37Ra *M. tuberculosis,* were also used. Cytotoxicity was assessed using a colorimetric assay against Vero kidney, human hepatoma and murine macrophage cells. Four out of 15 crude acetone extracts showed significant antimycobacterial activity with minimum inhibitory concentration varying from 50 to 100 µg/mL. *Searsia undulata* had the highest activity against most mycobacteria, followed by *Protorhus longifolia. M. fortuitum* was the strongest predictor of activity against multidrug-resistant TB (correlation coefficient = 0.65). Bioautography against *M. aurum* and *M. fortuitum* worked well as indicators of the Rf values of active compounds yielding strong zones of inhibition. The leaf extracts of *S. undulata* and *P. longifolia* had more than ten different antimycobacterial compounds and had low cytotoxicity with LC_{50} values above 100 µg/mL.

Keywords: Anacardiaceae; MDR-*M. tuberculosis*; antimycobacterial; cytotoxicity; selectivity.

BACKGROUND

Tuberculosis occurs in humans, cattle, wild animals and many other domesticated species. The *Mycobacterium tuberculosis* complex comprises *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canetti*, *Mycobacterium pinnipedii*, *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium mungi*, *Dassie bacillus*, *Oryx bacillus* and the attenuated *M. bovis* Bacille Calmette–Guerin (BCG) vaccine strain. With the exception of BCG, these species are pathogenic and can cause tuberculosis (TB) in mammalian hosts (Kabongo-Kayoka *et al*., 2015).

Despite the fact that TB can be cured, this disease remains a worldwide public health threat because of the emergence of multi and extremely drug resistant strains and subsequent poor response to current antibiotics. One-third of the world's population is infected with *M. tuberculosis* and is hence at risk of developing active TB. An order of 9.6 million people every year develops TB, and 1.5 million die from the disease. This includes 0.4 million TB deaths among HIV positive people (World Health Organization (WHO), 2015). From the 9.6 million, only 12% were HIV positive, and it was also estimated that 480 000 cases of multidrug-resistant TB (MDR-TB) have occurred in 2014. This confirms that TB alone is still a major challenge.

There is currently no effective vaccine to protect against TB. In humans, control of the disease relies heavily on detecting infectious cases and treating them for at least 6 months with a combination of antibiotics. The course of antibiotics involves the administration of first-line anti-TB drugs, which are combinations of isoniazid (INH), rifampicin (RIF), pyrazinamide and ethambutol for 2 months to kill the rapidly growing bacteria. The treatment is continued in the next 4 months with a combination of INH and RIF because of their sterilizing activity to eliminate bacilli that are dormant in the macrophages, or slow growers (Rivers and Mancera, 2008). Toxicity associated with these first-line drugs and the long duration of treatment has led to low patient compliance, giving rise to drug-resistant strains. Multidrug-resistant mycobacteria are resistant to INH and RIF, whereas extremely drug-resistant mycobacteria are resistant to second-line drugs such as fluoroquinolones and to at least one injectable drug in addition to INH and RIF. Hence, there is a need to find new anti-tubercular agents with novel modes of actions (Chinsembu, 2016).

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Transboundary and Emerging Diseases

Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Novel *Mycobacterium avium* Complex Species Isolated From Black Wildebeest (*Connochaetes gnou*) in South Africa

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5 Department of Emerging Infectious Diseases, Graduate School of Medicine, Tohoku University, Sendai, Japan
6 Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa
7 Department of Medical Microbiology, University of Pretoria, Pretoria, South Africa
8 National Health Laboratory Service, Pretoria, South Africa

Keywords:
non-tuberculous mycobacterium; *Mycobacterium avium* complex; *M. intracellulare*; phylogenetic analyses; black wildebeest

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doi:10.1111/tbed.12460

Summary

A study was undertaken to isolate and characterize *Mycobacterium* species from black wildebeest suspected of being infected with tuberculosis in South Africa. This led to the discovery of a new *Mycobacterium avium* complex species, provisionally referred to as the Gnou isolate from black wildebeest (*Connochaetes gnou*). Sixteen samples from nine black wildebeest were processed for *Mycobacterium* isolation. Following decontamination, samples were incubated in an ordinary incubator at 37°C on Löwenstein–Jensen slants and in liquid medium tubes using the BACTEC™ MGIT™ 960 system, respectively. Identification of the isolate was carried out by standard biochemical tests and using the line probe assay from the GenoType® CM/AS kit (Hain Lifescience GmbH, Nehren, Germany). The DNA extract was also analysed using gene sequencing. Partial gene sequencing and analysis of 16S rRNA gene, and 16S-23S rRNA (ITS), *rpoB* and *hsp65* and phylogenetic analyses by searching GenBank using the BLAST algorithm were conducted. Phylogenetic trees were constructed using four methods, namely Bayesian inference, maximum likelihood, maximum parsimony and neighbour-joining methods. The isolate was identified as *Mycobacterium intracellulare* using the GenoType® CM/AS kit and as *Mycobacterium avium* complex (MAC) by gene sequencing. The gene sequence targeting all the genes, ITS, 16S rRNA, *rpoB* and *hsp65* and phylogenetic analyses indicated that this isolate presented a nucleotide sequence different from all currently published sequences, and its position was far enough from other MAC species to suggest that it might be a new species.

Background

In late 2006, animals from a commercial game reserve farm in Mpumalanga province in South Africa were harvested for game meat exportation. During meat inspection, the animal carcasses showed lesions suspicious of tuberculosis which was supported by histopathological results. The exact cause of the disease was not determined, and the farm was put under quarantine for suspected bovine tuberculosis.

In February 2009, 158 animals were harvested. A high number of animals (N = 135) showed gross-visible tuberculosis-like lesions, and lesions from six animals processed for mycobacterial cultures yielded non-tuberculous mycobacteria.